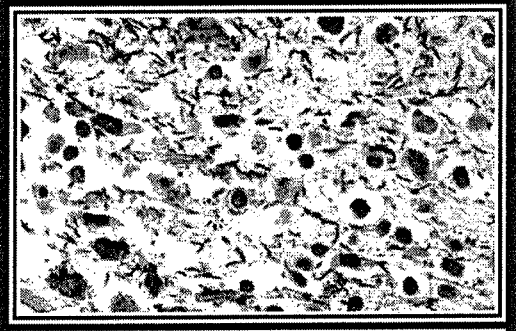
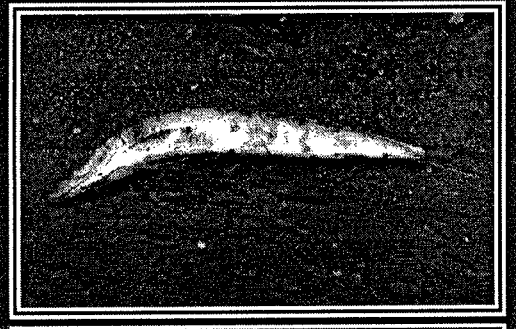
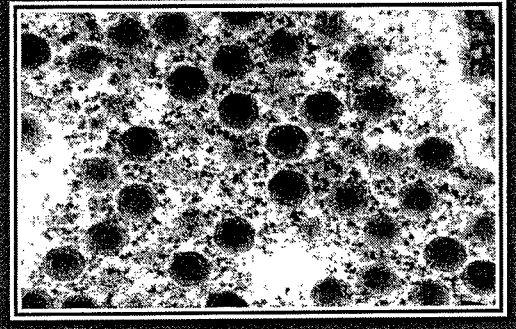
Appendix B

Fish Health Section BLUE BOOK, 2007 Edition

Fish Disease, Diagnosis and Treatment, Noga 2000







Fish Health Section
BLUE BOOK

Preface 2007

Citation Guidelines

Section 1: Diagnostic Methods

Section 2: Inspection Methods

Section 3: Quality Assurance/
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Help

Preface

This new 2007 edition of the American Fisheries Society, Fish Health Section Blue Book contains important updates on Viral Hemorrhagic Septicemia Virus in both the Diagnostic and the Inspection Sections. Information on susceptible species, appropriate cell lines for testing and PCR primers for confirmation have been updated. **The primer sequences for VHSV RT-PCR have been modified compared to those provided in previous editions of this manual. The new sequences now match the OIE (2006) VHSV primers. Also in this edition, the primer sequences for the IHNV RT-PCR assay have been modified from those in previous editions of this manual.** Additionally, the Diagnostic Manual chapter on Infectious Pancreatic Necrosis Virus has been updated and four new chapters including Goldfish Herpesviral Hematopoietic Necrosis Virus, Largemouth Bass Virus Disease, Necrotizing Hepatopancreatitis of Penaeid Shrimp, and Taura Syndrome Virus of Penaeid Shrimp have been added. The shrimp virus chapters are in a new portion of the Diagnostic Section entitled "Diseases of Crustaceans". The date of the most recent revision of each section is at the bottom of each page. **No 2006 Edition of the Blue Book was produced.**

As in the 2005 edition of the AFS-FHS Blue Book, a major feature is the Standard Procedures for Aquatic Animal Health Inspections. This Section is a collaborative effort between the U. S. Fish and Wildlife Service and the Fish Health Section and identifies specific procedures recommended for the detection of certain pathogens of regulatory concern. This Section replaces Chapter 1 of the 4th Edition, and is greatly expanded for the purpose of providing information for persons performing inspections for the inter- and intra-state movement of fish. An identical document also appears in the USFWS Handbook. The USFWS and FHS are committed to an annual review and revision of these procedures according to a formal protocol detailed in the Inspection Section, Appendix 1. While some pathogens are included in both the Inspection and Diagnostic sections, the information provided and the technical approaches are different. The individual pathogen chapters in the Diagnostic Section cover topics like geographic and host range, and epizootiology, which are not included in the inspection chapter. They also describe a wider range of diagnostic methods, recognizing that these methods are used for much broader purposes than inspections, and that there are many pathogens of importance to diagnosticians but that are not often included in formal fish health inspections. You will also note that the 2007 Blue Book has no general editor. The "Citation Guidelines" section has the suggested method for citing chapters in the Blue Book. This format provides the authors with recognition for their efforts and also allows for different publication dates within a volume.

This digital edition of the Blue Book is available as a subscription, somewhat like a journal published annually. This was done because the new inspection portion is required to be updated annually and because this new format provides an opportunity to make timely additions and updates to the rest of the Blue Book. Another great advantage is that it is simple to incorporate more images and even movies, which appear now in two chapters. Subscriptions for the new Blue Book will run for five years, with the understanding that publication options are rapidly changing and we will need to re-evaluate this process during this period.

Production of this 2007 edition required hundreds of volunteer hours contributed by the members of the Inspection Section Committees, by the FHS Technical Standards Committee, and by authors of Diagnostic Chapters. We hope readers and users of this document find it useful and will participate in the process of improving it in the years to come.

A4.1 Citation Guidelines

In 2003, the 4th Edition of the Blue Book (1994) underwent a major revision to include the joint FHS/USFWS Fish Inspection Handbook and many new or updated diagnostic chapters. In addition, the entire document was converted to a digital format for distribution on CDs. These revisions made it necessary to change the way that the Blue Book is structured and cited.

Within the Blue Book, all of the chapters in both sections now have a footer that tells when the chapter was last revised. For purposes of citation, chapters in this document are cited as chapters in a multi-author/multi-chapter book. The editions will no longer be numbered (as in "4th edition") but will now be described only by the publication date ("2007 edition"). Chapters in the Diagnostic Section are credited to the individual authors that appear on the title page of each chapter. The year cited after the author's name should be the year in which the chapter was last revised. For the Inspection Section, the section should be cited as a single chapter with the USFWS and AFS-FHS as authors and the same date as the Blue Book edition. Specific examples for citing chapters from this new edition of the Blue Book are below.

Section 1 (the Diagnostic Section) chapters in this document may be cited as follows:

Mitchell, A., and A. Goodwin. 2004. Centrocestiasis (gill trematode disease). *In* AFS-FHS (American Fisheries Society-Fish Health Section). FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Johnson, K. 1994. Lernaeid parasitism. *In* AFS-FHS (American Fisheries Society-Fish Health Section). FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Section 2 (the Inspection Section) of this document may be cited as:

USFWS and AFS-FHS (U.S. Fish and Wildlife Service and American Fisheries Society-Fish Health Section). 2007. Standard procedures for aquatic animal health inspections. *In* AFS-FHS. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

Section 3 (the Quality Assurance/Quality Control Section) of this document may be cited as:

AFS-FHS (American Fisheries Society-Fish Health Section). 2007. Model Quality Assurance/Quality Control Program For Fish Health Laboratories, 2007 edition. AFS-FHS, Bethesda, Maryland.

The Blue Book in its entirety may be cited as:

AFS-FHS (American Fisheries Society-Fish Health Section). 2007. FHS blue book: suggested procedures for the detection and identification of certain finfish and shellfish pathogens, 2007 edition. AFS-FHS, Bethesda, Maryland.

1.1 General Procedures for Bacteriology

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I. Sampling and Handling of Samples

For the detection and identification of bacterial pathogens in populations of fish showing disease signs, ideal samples are multiple (five or more) moribund fish or those showing clinical signs typical of the disease outbreak. For the detection of subclinical infections in populations of asymptomatic fish, larger sample numbers may be necessary (see Section 2, 2.2.D "Sample Number"). Fish that are found dead at the time of sampling are not acceptable for bacteriological examination, unless they are known to be very fresh. Contaminating bacteria can grow quickly in dead fish, particularly in warm water. Fish usually are not pooled for testing, although this is sometimes done for certain assays when fish are very small.

The selection of tissue samples for bacteriological assays varies depending on the pathogen suspected; the reader is referred to chapters on specific bacterial diseases. Organs most commonly tested include the kidneys and portions of any organ with visible lesions, although other organs such as the spleen or brain, and fluids such as blood plasma or ovarian fluid, are used for the detection of certain bacterial infections.

Samples for attempted culture of bacteria must be taken aseptically. If samples are to be taken from internal organs, disinfection of the body surface is recommended before incisions are made to expose the organs. Sterile tools must be used for making incisions and removing samples for culture. Samples intended for Gram stains or immunological tests also should be taken aseptically to prevent contamination with bacteria or antigens from extraneous sources. Homogenization of samples prior to culture or immunological testing may enhance the detection of bacteria or their antigens.

If samples cannot be inoculated immediately onto appropriate culture media, they may be stored on ice for up to 24 hours. Samples for culture should not be frozen. For the storage of samples intended for immunological tests, refer to the chapters on specific bacterial diseases.

II. Bacteriological Media and Test Reagents

A. Media

Specialized media for the detection of certain bacteria are referenced in the appropriate chapters. The following media commonly are used for the isolation of fish pathogens:

1. **Trypticase (or Tryptic) Soy Agar (TSA)**
   This is a commonly used medium for routine isolation and culture of many fish pathogens, and it is available from commercial sources.

2. **Brain-Heart Infusion Agar (BHIA)**
   This medium is used for routine isolation and culture of many fish pathogens, and it is available from commercial sources.

3. **Blood Agar (BA)**
   This medium is used for routine isolation and culture of many fish pathogens, and is also used for the visualization of hemolysis (see below). It is available from commercial sources, or can be prepared by the addition of sterile defibrinated blood (usually sheep, horse, or rabbit) at a concentration of 5% (volume/volume) to a sterile base medium such as TSA or BHIA, when the base medium has cooled to 48 to 50°C.

4. **Tryptone Yeast Extract Salts Agar (TYES) Medium (Holt et al. 1989)**
   This medium is used for growth of *Flavobacterium psychrophilum.* This medium is a modification of one reported by Fujihara and Nakatani (1971) for growth of *F. columnare.*

Tryptone (Difco)	0.4%
Yeast extract (Difco)	0.04%
$MgSO_4.7H_2O$	0.05%
$CaCl_2.2H_2O$	0.05%
Agar	1.0%

   Adjust pH to 7.1-7.3.

   TYES plus skim milk agar is a recent modification that has the advantage of observing clearing in the medium around the *F. psychrophilum* colonies. For this medium sterile skim milk is added to TYES agar after the medium is cooled to 45 to 50°C just prior to pouring the plates. One mL of a 20% sterile skim milk solution is added for every 100 mL of TYES agar. The 20% skim milk is prepared by dissolving the skim milk powder in distilled water on a hot plate, then sterilizing the solution in 8 to 10 mL quantities.

5. **Cytophaga Agar**
   This medium (Anacker and Ordal 1959) is used for the isolation of flavobacteria. The formula is:

Tryptone	0.5 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.2 g
Agar	11.0 g

Distilled water to	1000.0 mL

Adjust pH to 7.2.

The growth of flavobacteria can be enhanced by the addition of 2 to 5% fetal bovine serum to this medium.

6. **Hsu-Shotts Medium.**
This medium (Bullock et al. 1986) is used for the isolation of flavobacteria; the addition of neomycin sulfate enhances the isolation of these bacteria by suppressing the growth of many other bacteria. The formula is:

Tryptone	2.0 g
Yeast extract	0.5 g
Gelatin	3.0 g
Agar	15.0 g
Distilled water to	1000.0 mL

After it is autoclaved, the medium is cooled to 45°C, and filter-decontaminated neomycin sulfate is added to a final concentration of 4.0 mg/L.

Formulations for routine bacteriological media can be found in manuals on bacteriological methods such as MacFaddin (1985), Difco Laboratories (1998), Baron and Finegold (1990), and Murray (1999).

B. Test Reagents and Procedures for Phenotypic Characterization of Bacteria

The following are some tests commonly employed in the presumptive identification of bacteria isolated from fish. Commercially prepared media and reagents are available for biochemical tests. Detailed instructions on their preparation and use are also included in bacteriology manuals, and some descriptions are included in Section 2, 3.7 Reagents, Media, and Media Preparation and Section 2, 3.8 Bacterial Identification Techniques. Miniaturized bacterial identification systems that include many of the tests are also available commercially; their use for fish pathogen identification is further discussed in Section 2, 3.8.D.2 "Commercial Identification System."

1. **Gram Stain**
This test is important for the broad classification of bacteria, and should be done on young (log-phase) cultures. Gram stain procedures are described in Section 2, 3.8.A.1 "Gram Stain." Nonstain methods are available to aid in the determination of the true Gram stain reaction of problem organisms. One of these is the potassium hydroxide (KOH) test. The cell walls of gram-negative bacteria are broken down by KOH, and viscid chromosomal material is released. This causes the suspension to become thick and stringy. Procedures for the KOH test are described in Section 2, 3.8.A.2 "3% Potassium Hydroxide." Fluorescent stains for differentiating gram-positive and gram-negative bacteria can also be used and are available from commercial sources.

2. **Acid-Fast Stain**
   This test is used to distinguish gram-positive (or gram-variable) bacteria with cell walls that contain mycolic acids (long-chain, multiple cross-linked fatty acids) from other gram-positive bacteria. Mycobacteria (and to a lesser extent, *Nocardia*) stain acid fast.

3. **Motility**
   For most bacteria (i.e., those motile by flagella), wet mounts (hanging drops) are prepared from log-phase cultures with TSB (trypticase soy broth or tryptic soy broth) as the suspending medium. If microscopic examination of wet mounts (hanging drops) gives equivocal or negative results, motility can be evaluated further by stab-inoculating tubes of commercially available semi-solid motility test medium (Difco Motility Test Medium or equivalent). The use of the hanging drop method and motility test medium for motility evaluations are described in Section 2, 3.8.C "Motility."

   For flavobacteria, an agar block motility test can be done by excising a 5-mm square block of agar supporting a suspected flavobacterial colony, placing the block (colony side up) on a glass slide, and covering the block with a cover glass. The margin of the colony is examined with a microscope at about 400X magnification for evidence of gliding or creeping motility.

4. **Cytochrome Oxidase Test**
   This test indicates the presence of the enzyme cytochrome oxidase, an iron-containing porphyrin enzyme that participates in the electron transport mechanism and in the nitrate metabolic pathways of some bacteria. The test is used to initially characterize gram-negative bacilli. Cytochrome oxidase procedures are discussed in Section 2, 3.8.B "Cytochrome Oxidase."

5. **Carbohydrate Utilization Tests**
   Preparations of basal media to which specific carbohydrates are added are used to differentiate bacteria according to their patterns of carbohydrate utilization. Utilization of a specific carbohydrate causes a pH change, which is detected by a pH indicator in the medium. To determine if organisms metabolize carbohydrates oxidatively or fermentatively, OF medium is used. Gas production can be detected in some media. Further discussions of OF basal medium and certain carbohydrate utilization tests are included in Section 2, 3.7.B.4 "Oxidation/Fermentation (OF) Medium," Section 2, 3.8.D.1.a "Glucose Fermentation," and Section 2, 3.8.D.1.e "Carbohydrate Utilization (MacFaddin 1980)."

6. **Triple Sugar Iron (TSI) Agar**
   Triple sugar iron agar is frequently used during the initial identification of gram-negative bacilli, particularly members of the Enterobacteriaceae. This medium can detect three primary characteristics of a bacterium: the ability to produce gas from the fermentation of sugars, the production of large amounts of hydrogen sulfide (H_2S) gas, and the ability to ferment glucose, lactose and sucrose. The interpretation of results is described in Section 2, 3.8.D.1.b "Triple Sugar Iron (TSI)." Lead acetate paper, a more sensitive indicator of H_2S production, is sometimes used in conjunction with TSI or other media containing available sulfur compounds.

7. **Single Substrate Utilization Tests**
   Certain bacteria can be characterized by their ability to grow in the presence of a single compound. Common substrates that can fulfill this function and are useful for the differentiation of bacteria are citrate, malonate, and acetate. Growth on an agar slant containing the substrate of interest (with or without a pH indicator) is used as the end point of the test. The malonate test is described in and Section 2, 3.8.D.1.g "Malonate Test,"

respectively.

8. **Indole Test**
   This is a test for the enzyme tryptophanase, and is used in the characterization of gram-negative bacteria, particularly Enterobacteriaceae. Bacteria that produce this enzyme can degrade the amino acid tryptophan into pyruvic acid, ammonia, and indole. Indole is detected by its combination with an indicator aldehyde to form a colored product. The indole test is described in Section 2, 3.8.D.1.d "Indole Test."

9. **Decarboxylase/Dihydrolase Reactions**
   These are tests for enzymes that degrade certain amino acids by decarboxylation or dihydrolation. Common tests used in the identification of fish pathogenic bacteria include those for arginine dihydrolase/decarboxylase (ADH), lysine decarboxylase (LDC), and ornithine decarboxylase (ODC). The lysine decarboxylase test is described in Section 2, 3.8.D.1.f "Decarboxylase Test (Lysine)."

10. **Hydrolysis Tests**
    These tests are used to differentiate bacterial species according to their abilities to produce certain hydrolyzing enzymes. The products of hydrolysis are detected by some visual reaction. Substrates commonly used in the characterization of fish pathogenic bacteria are urea (for urease activity), gelatin (for gelatinase activity), esculin (for esculin hydrolysis), and starch (for starch hydrolysis). The gelatinase test and esculin test are described in Section 2, 3.8.D.1.c "Gelatinase" and Section 2, 3.8.D.1.h "Esculin Test," respectively.

11. **DNAse Test**
    Extracellular nucleases are produced primarily by the same bacterial species that produce the protease gelatinase. Commercially produced agar media are available for this test.

12. **Phenylalanine Deaminase Test**
    This test determines the ability of an organism to deaminate the amino acid phenylalanine to yield indolepyruvic acid, and is used for grouping Enterobacteriaceae.

13. **Catalase Test**
    This test is used to detect the presence of the enzyme catalase, which catalyzes the liberation of oxygen and water from hydrogen peroxide. The test reagent (3% hydrogen peroxide) is readily available. Because red blood cells in blood agar media contain catalase, weak false positive reactions can be obtained with colonies taken from these media. It is recommended that control catalase tests be performed with a small loopful of blood-containing agar on the same slide with the organism; a strong positive reaction from the organism can be distinguished from a weak positive reaction from the medium alone.

14. **Bile Solubility Test**
    Streptococcus pneumoniae can be distinguished from other alpha hemolytic streptococci (see below) by this test. *Streptococcus pneumoniae* possesses an autocatalytic enzyme that normally functions to lyse the cell wall during cell division; colonies of this organism also will autocatalyze within 30 minutes after exposure to the surfactant sodium deoxycholate, one of the major components of bile. Other alpha hemolytic streptococci lack such an active enzyme and will not dissolve in bile. The bile solubility test may not work with old colonies of *S. pneumoniae*, which may have lost their active enzyme.

15. **Nitrate Reduction Test**
    This test distinguishes between bacteria that cannot utilize nitrate (NO_3) as a nitrogen source,

and those that can reduce nitrate to nitrite (NO_2) or a product beyond nitrite.

16. Antimicrobial Sensitivity Tests

Patterns of sensitivity to various antimicrobial reagents are sometimes used in the characterization of fish pathogenic bacteria. Most commonly, sensitivity to novobiocin is used to distinguish *Vibrio* spp. from motile *Aeromonas* spp. The novobiocin disk (5 µg concentration) is applied to a TSA plate that has been surface-seeded uniformly with the organism under test. After incubation at 20 to 22°C for 16 to 24 hours, a sensitive organism shows a clear zone of inhibition around the disk. Because TSA is widely used for the primary isolation of many fish pathogenic bacteria, this is the medium generally used for novobiocin sensitivity tests performed to differentiate *Vibrio* spp. from motile *Aeromonas* spp. However, disk diffusion tests performed to determine the sensitivity patterns of bacterial isolates to a battery of compounds are done on Mueller-Hinton agar (the para-aminobenzoic acid present in TSA interferes with the action of sulfonamides). Procedures for the conduct and interpretation of disk diffusion antimicrobial susceptibility tests are described in more detail in manuals on bacteriological procedures.

17. Vibriostatic Agent 0/129 Sensitivity Test

Sensitivity to the vibriostatic agent 0/129 (2,4-diamino-6,7-diisopropylpteridine phosphate, available from Sigma Chemical Co.) is used to differentiate *Vibrio* spp. from motile *Aeromonas* spp. The sensitivity disks are prepared as follows: Dissolve 0/129 in acetone at 0.1% (weight/volume). Saturate Whatman antibiotic filter paper disks (6 mm) with the 0/129 solution, and drain off the excess. Dry the disks at 37°C, and store them in a tightly sealed bottle at 4°C. Control disks (saturated with acetone only) also should be prepared. For 0/129 sensitivity tests, TSA plates are surface-seeded with bacteria, test and control disks are applied to the plates, the plates are incubated, and results are interpreted in the same manner as for novobiocin sensitivity tests. Novobiocin sensitivity and 0/129 sensitivity tests can be conducted on the same plate.

18. P Disk Test

The P disk (optochin resistance) test is used to identify strains of *Streptococcus pneumoniae*. Disks impregnated with 5 µg of optochin (ethyl hydrocupreine) are placed on inoculated blood agar plates. *Streptococcus pneumoniae* is not optochin resistant and a zone of inhibition will develop around the disk. The isolate is resistant to optochin if the zone of inhibition is greater than 14 mm for a 6-mm disc or 16 mm for a 10-mm disk. Correct interpretation of the optochin zone requires a confluent lawn of bacterial growth. If the growth is too light, an erroneously large zone (false susceptibility) may be observed.

19. LAP Test

The LAP test detects the presence of leucine aminopeptidase (LAP). Among the LAP-positive bacteria are the lactococci, enterococci, and several streptococci, but the ß-hemolytic streptococci and aerococci are LAP-negative. The test is performed using commercially available discs that are impregnated with leucine-ß-naphthylamide. The LAP enzymatic activity releases ß-naphthylamine, which turns red on the addition of p-dimethylaminocinnamaldehyde.

20. Characterization of Hemolysis

Patterns of hemolytic action of bacteria on erythrocytes in blood-containing media (usually BA) are used in the identification of some bacteria, principally streptococci. The major types of hemolysis are: α (alpha), consisting of an indistinct zone of incomplete lysis of erythrocytes around the bacterial colony, accompanied by a greenish to brownish

discoloration of the medium; and β (beta), a clear, colorless zone around the bacterial colony, in which the erythrocytes have undergone complete destruction; and γ (gamma), no hemolysis.

21. CAMP Test

The CAMP test is used to identify Lancefield Group B streptococci (see Immunological Procedures below). Group B streptococci produce a protein-like substance called the "CAMP factor," which can act synergistically with the beta toxin produced by certain strains of *Staphylococcus aureus*, resulting in an enhanced zone of hemolysis around the streptococci. The test is performed with an appropriate strain of *S. aureus*, or with commercially available CAMP factor-impregnated filter paper disks.

22. Simmons Citrate Test

Organisms that are able to use citrate as the sole source of carbon for metabolism and growth are able to grow on simmons citrate agar. In the process of metabolizing citrate, bacteria produce alkaline conditions in the medium. Simmons citrate agar contains the pH indicator bromothymol blue, which is green under acidic conditions, and royal blue when the medium becomes alkaline.

Magnesium sulfate	0.20 g
Monoammonium phosphate (NH4H2PO4)	1.00
Dipotassium phosphate (K2HPO4)	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Agar	15.00
Bromthymol blue	0.08
Final pH 6.9 ± 0.2 at 25°C	

Heat gently with occasional agitation. Boil 1 to 2 minutes until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 minutes at 121°C. Before medium solidifies, incline tubes to obtain 4 to 5 cm slants and 2 to 3 cm butts. Final pH, 6.8 ± 0.2.

III. Immunological Procedures

Confirmation of the identity of most fish pathogenic bacteria is based on serological tests. Most common among these are the fluorescent antibody test and the slide or microtiter agglutination test. These and certain other techniques developed for the identification of bacteria in culture, or for the detection and identification of bacteria directly in fish tissues or body fluids, are discussed briefly below. The specificity of these and other immunological tests for the identification of fish pathogens will depend on the specificity of the antibodies used.

A. Fluorescent Antibody Test (FAT)

Both direct and indirect FATs have been developed for the identification of bacterial fish pathogens. Antibodies for specific bacteria are available from commercial sources, or from certain research laboratories. Fluorochrome-conjugated antibodies for the indirect FAT are readily available from commercial sources. Most commonly, the FAT is used to confirm the identity of bacteria isolated in culture. For some pathogens, notably *Renibacterium salmoninarum*, the FAT is used for the detection and identification of bacteria directly in fish

tissues or body fluids. Descriptions of direct and indirect FAT procedures are included in Section 2, 3.8.E "Fluorescent Antibody Test (FAT)." The reader is also referred to chapters on specific pathogens for modifications of the FAT for use on fish tissue and body fluid samples.

B. Agglutination Tests

The identity of many bacterial fish pathogens can be confirmed by agglutination reactions with specific antisera. Certain bacterial species exhibit a tendency to autoaggregate spontaneously and are therefore difficult to examine by agglutination tests. Such bacteria may be subjected to pretreatment procedures to prevent autoaggregation, or they may be tested by alternative procedures such as latex bead agglutination or staphylococcal coagglutination. The reader is referred to chapters on specific bacterial pathogens for modifications to agglutination tests. Principles and procedures for some agglutination techniques are also discussed by Roberson (1990).

1. Slide Agglutination Test

This test is used for rapid confirmatory identification of bacteria grown in culture.

a. Clean glass microscope slides and mark circular divisions (two circles per slide) with a wax crayon.

b. Prepare a suspension of one or more colonies of the unknown bacterium in a small amount of physiological saline. To ensure a uniform suspension without clumps, it is best prepared in a small test tube rather than directly on the slide. The bacterial suspension should be standardized to a known concentration of particulate materials; i.e. a McFarland standard #3 or a spectrophotometer reading of 40% T at 645 nm. If this cannot be done, preparation of a cloudy suspension approximating the color and density of skimmed milk is generally acceptable. Suspensions of positive controls (bacteria known to be the species of interest) and negative controls (known bacteria of an unrelated species) can be prepared in the same manner.

c. Place a drop of the unknown bacterial suspension in each of the two circles on a slide. Add a drop of the appropriate specific antiserum to one circle on the slide. Add a drop of normal serum to the other circle on the slide. Mix gently with an applicator stick, or rock the slide gently. Follow the same procedure for the control bacteria.

d. Observe the reactions immediately for agglutination (clumping), then incubate the slides at room temperature and observe after five and 10 minutes. Results can be checked with a microscope at low magnification.

For positive identification of the unknown bacterium, the following reactions should be observed:

Circle with unknown bacterium plus antiserum: agglutination

Circle with unknown bacterium plus normal serum: no agglutination

Circle with positive control bacterium plus antiserum: agglutination

Circle with positive control bacterium plus normal serum: no agglutination

Circle with negative control bacterium plus antiserum: no agglutination

Circle with negative control bacterium plus normal serum: no agglutination

2. **Microtiter agglutination test.** This test gives the titer of the antiserum used, in addition to confirmatory identification of the bacterium being tested. The test is done in 96-well microtiter plates. A constant bacterial concentration, but two-fold serial dilutions of antiserum are used. The procedure is described by Roberson (1990).

C. Lancefield Grouping of Streptococci

Most beta-hemolytic streptococci and some alpha-hemolytic or nonhemolytic streptococci possess specific carbohydrate cell wall antigens. These carbohydrate antigens are called streptococcal group antigens or Lancefield group antigens. In the past, serological typing of cell wall components was used to separate streptococci into species. Although recent DNA homology studies have shown that this is not possible, serological typing is still a useful aid for the identification of isolates. Group-specific sera (precipitating, agglutinating, and fluorescent-antibody sera) are commercially available for use with extracts, cell suspensions, and spent broth media. Beta-hemolytic streptococci are characteristically tested with group A, B, C, D, and F antisera. Alpha-hemolytic or nonhemolytic streptococci are usually tested with antisera to groups B, D, and N. Procedures for streptococcal antigen extraction and serotyping are described in bacteriological procedures manuals.

D. Other Immunological Tests

In addition to the tests described above, other immunological tests have been developed for the identification of bacteria in culture, or for the detection and identification of specific bacteria in fish tissues or body fluids. Included among these are such techniques as staphylococcal coagglutination, latex bead agglutination, counterimmunoelectrophoresis, and enzyme immunoassays. Of the enzyme immunoassays, the enzyme-linked immunosorbent assay (ELISA) has perhaps gained the widest use. An ELISA is designed to detect a specific substance in a complex mixture by binding that substance to an antigen- or antibody-coated surface. After binding has occurred, an enzyme-labeled antibody specific for the bound substance is applied. With the addition of appropriate reagents, the enzyme catalyzes a reaction that yields a colored end product, thus allowing detection and quantification of the bound substance. Various ELISAs have been developed for the detection of bacteria or bacterial products as well as viruses, drugs, hormones, toxins, carcinogens, and antibodies. The reader is referred to chapters on specific pathogens for further discussions of the ELISA and other immunological techniques.

IV. Nucleic Acid-based Identification Procedures

Nucleic-acid-based tests are now gaining favor for identification of certain bacterial fish pathogens. Among the early tests developed were nucleic acid probes designed to detect specific unique segments of DNA or RNA of the target bacterium. These techniques have been largely supplanted by a plethora of molecular tools known collectively under the name polymerase chain reaction (PCR). The PCR procedures are considered to have a greater potential for improving both the sensitivity and specificity for detection and identification of a target bacterium because of the enzymatic amplification steps incorporated in the tests. The reader is referred to chapters on specific bacteria for discussions of PCR procedures.

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1.2 Flow Chart for the Presumptive Identification of Selected Bacteria from Fishes*

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This revised key for the identification of bacterial pathogens of fish was developed by Dr. Emmett Shotts in 2001 and was designed to replace and simplify the key found in the 1994 edition of the Blue Book. This new key provides a broad perspective of strategies useful for the biochemical identification of bacterial fish pathogens and it will be helpful in both diagnostic work and in teaching. Users of these new tables must keep in mind that this is a presumptive key and thus it may lead to an incorrect identification of bacterial isolates having atypical biochemical characteristics or of species not included in this key. It is also important to note that some bacteria require special growth media or conditions for growth and that the initial isolations must be made using appropriate substrates and conditions. Diagnosticians should use this key as a helpful guide, but final diagnostic decisions should be based on more information provided in other specific bacteriology subchapters of this chapter or in Section 1, 1.1 General Procedures for Bacteriology.

Figure 1: Gram Stain

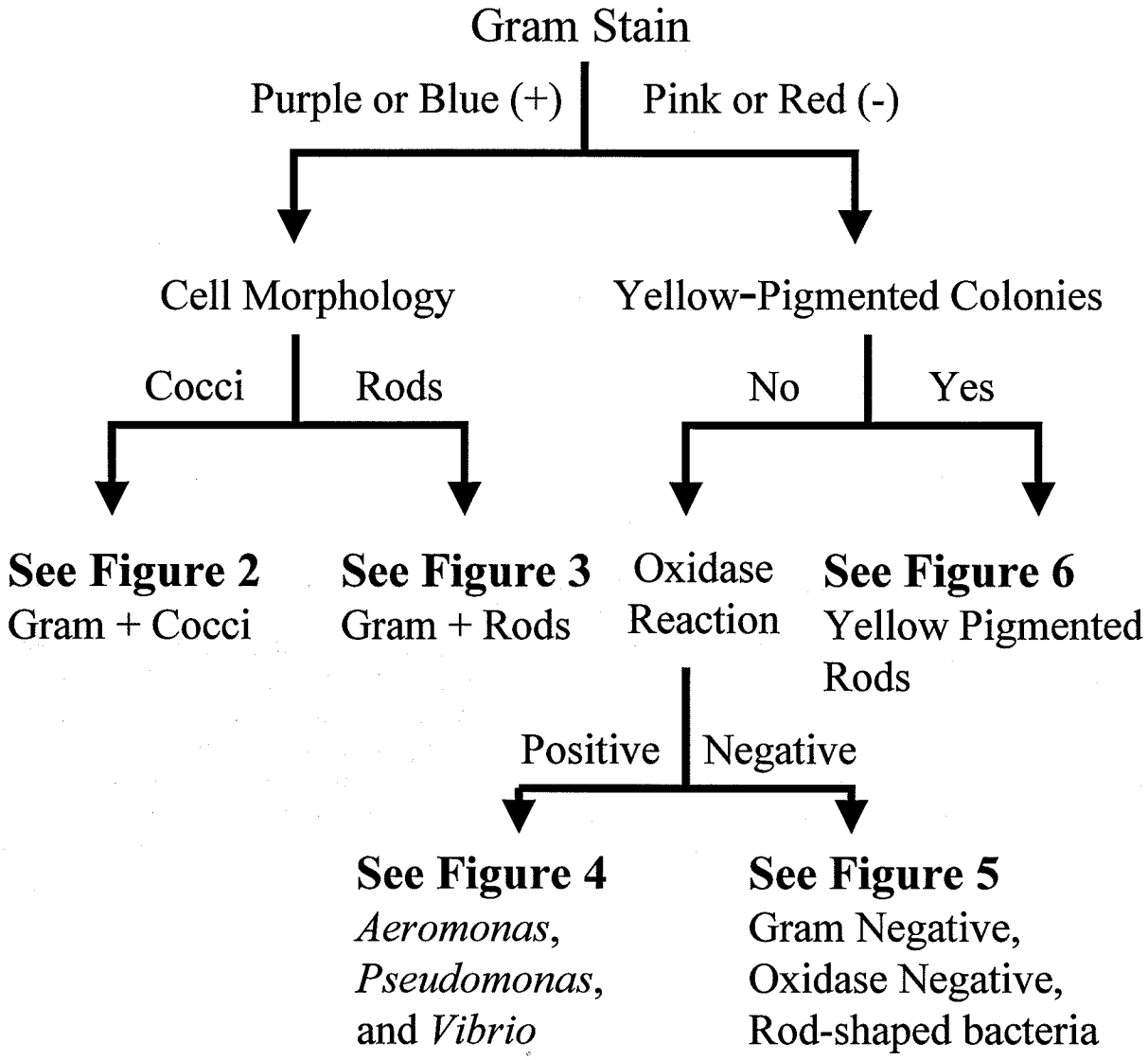
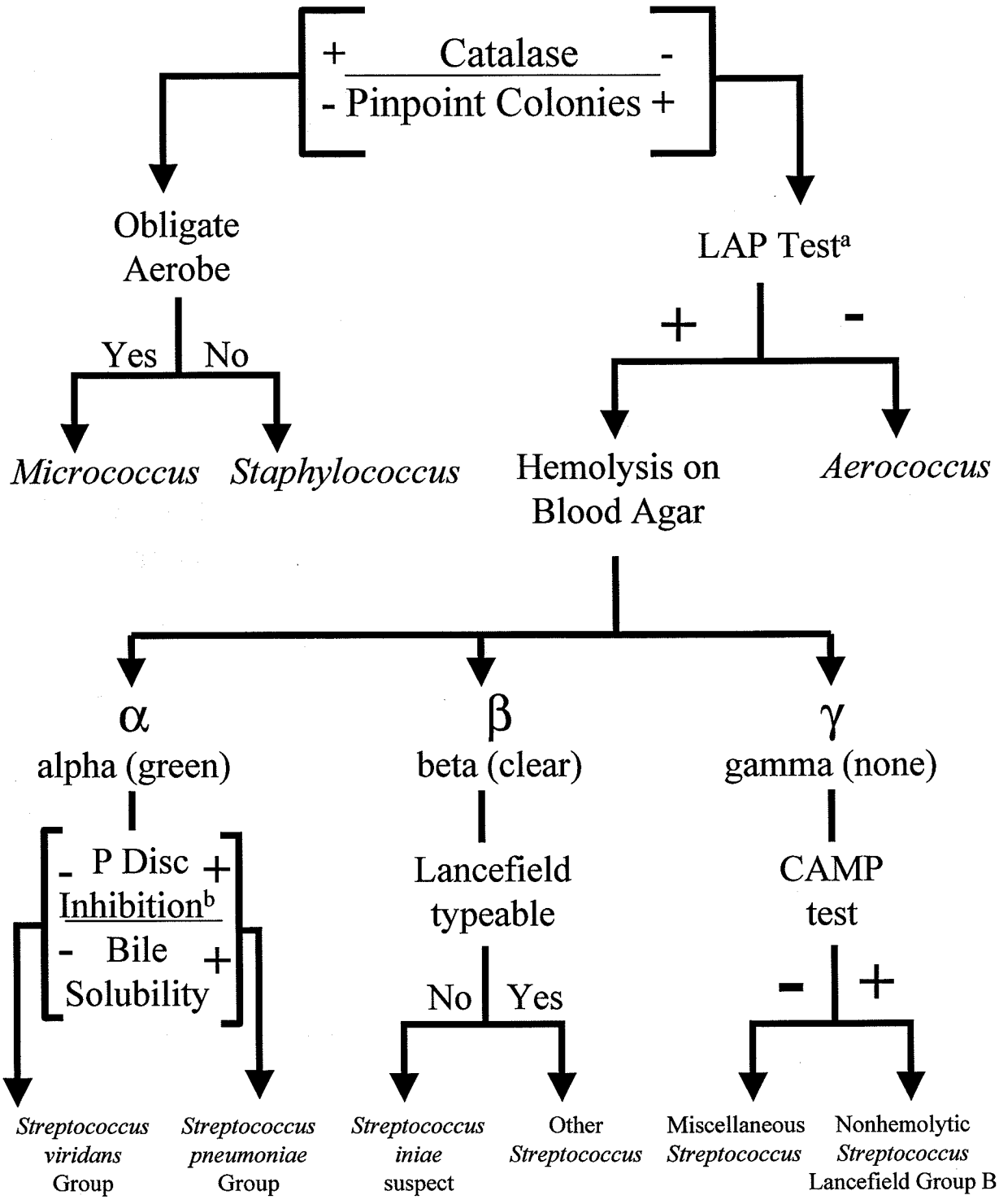


Figure 2: Gram Positive Cocci



a = leucine aminopeptidase test
b = ethyl hydrocuprein hydrochloride (5 μg) disc

Figure 3: Gram Positive Rods

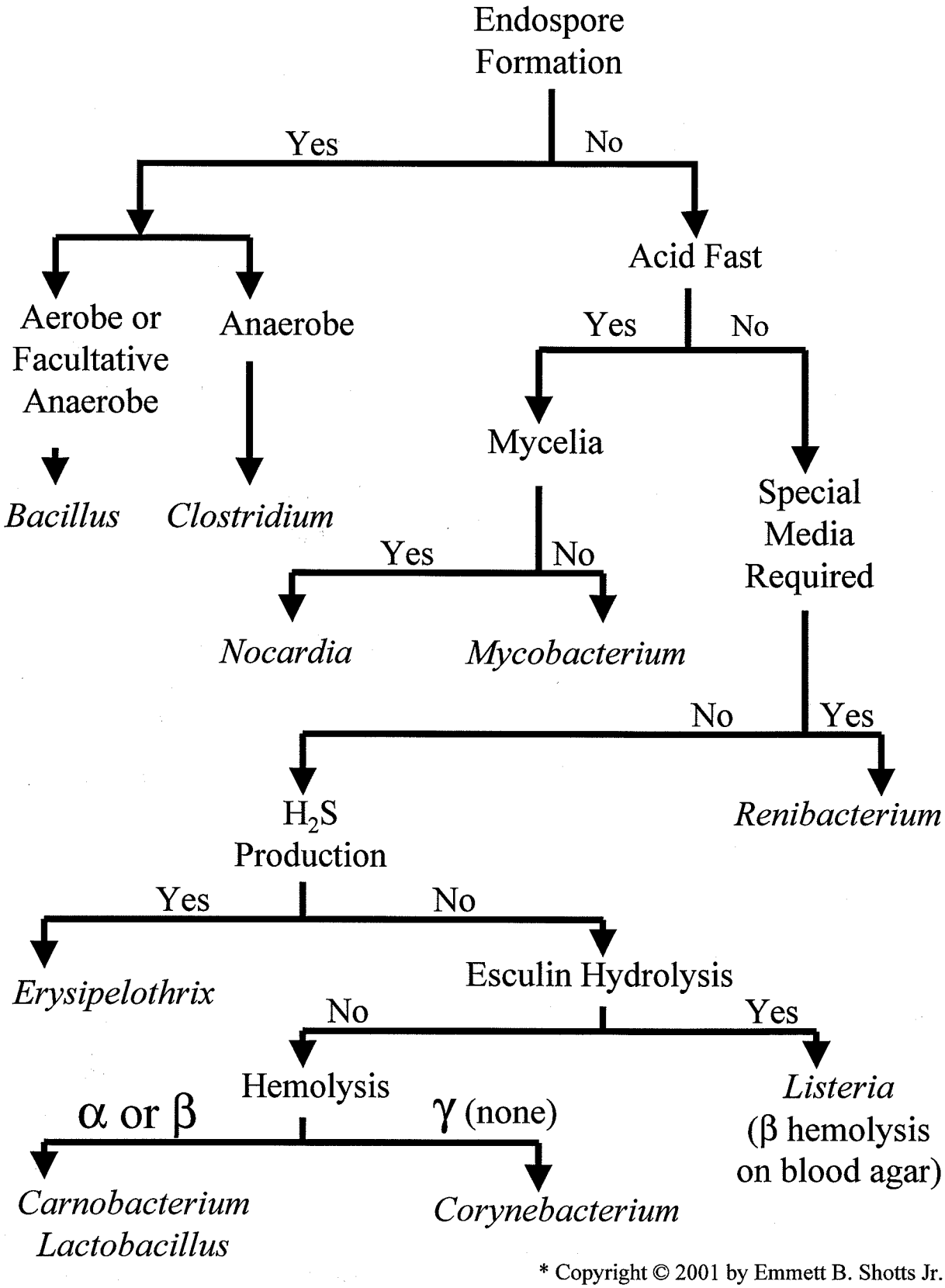


Figure 4: *Aeromonas*, *Pseudomonas*, and *Vibrio*

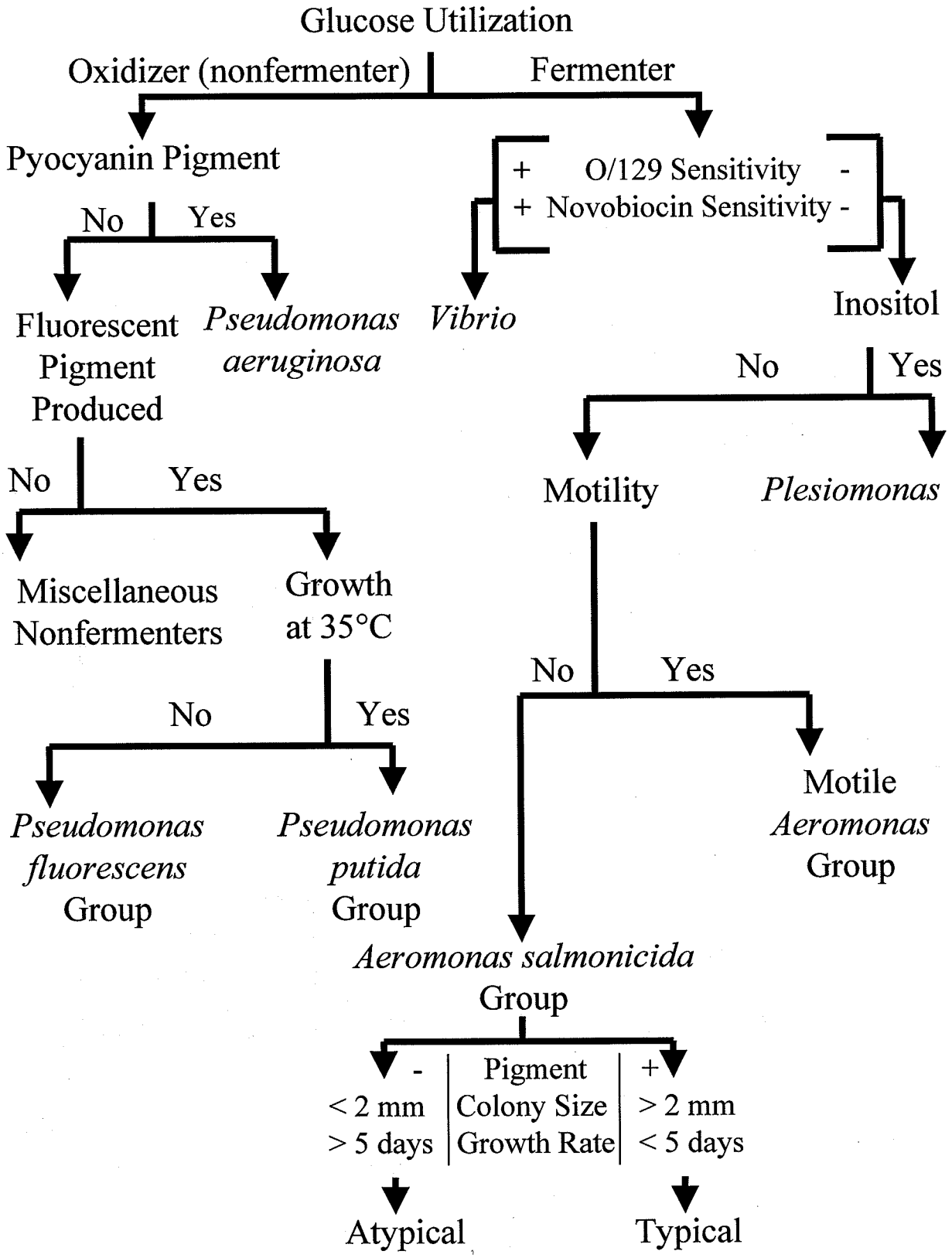


Figure 5:Gram Negative, Oxidase Negative, Rod-shaped bacteria

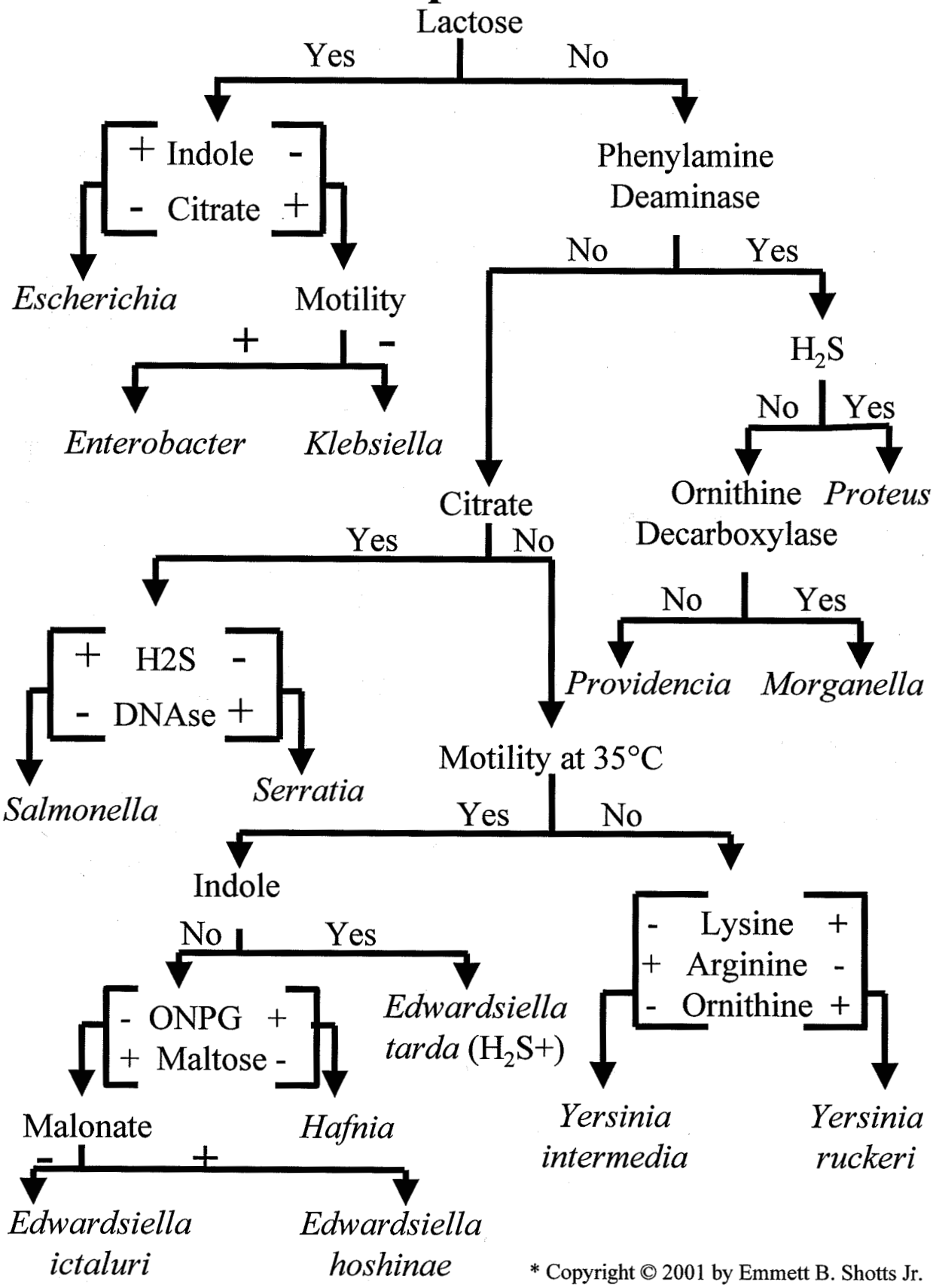
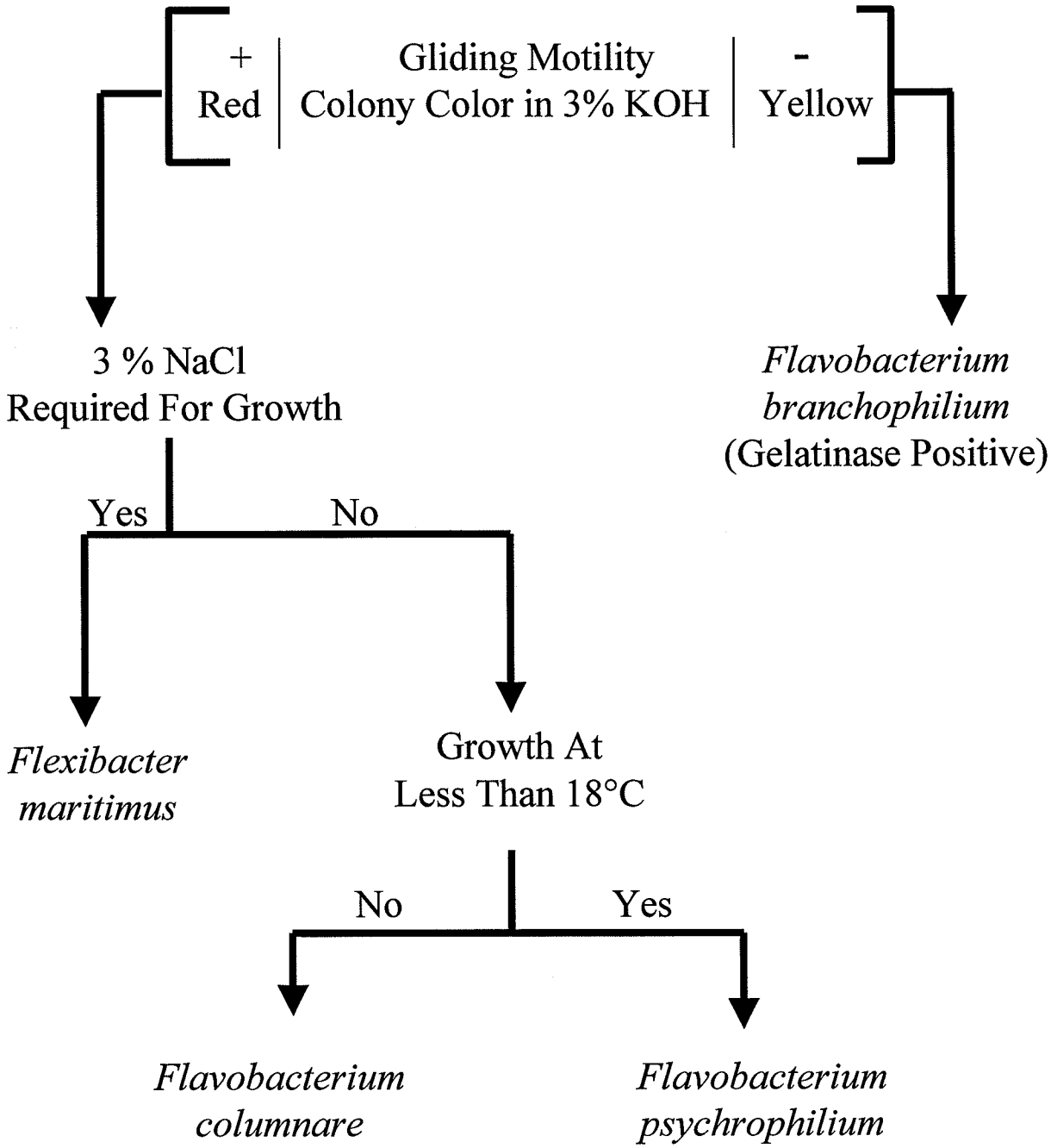


Figure 6: Yellow-Pigmented Rods



1.3 Bacterial Gill Disease

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A. Name of Disease and Etiological Agent

Bacterial gill disease, is caused by *Flavobacterium branchiphilum.* Other species of yellow pigmented, filamentous bacteria may also be involved.

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Worldwide, usually seen in fishes in aquaculture facilities.

2. **Host Species**
   All salmonids are probably affected; may occur in other fishes.

C. Epizootiology

Outbreaks of bacterial gill disease are associated with stressors such as crowding, low dissolved oxygen, high ammonia concentrations, and high feeding rates. Bacterial gill disease has been observed in all fish life stages.

The source of *Flavobacterium branchiphilum* is not known. The bacterium may be a common resident of water and sediment.

The incubation period for bacterial gill disease is variable because epizootics are dependent on the presence of the stressor. Bacterial gill disease has been induced experimentally in rainbow trout fingerlings in 10 to 14 days when the fish were subjected to poor environmental conditions. Fish less than 4.5 g are particularly susceptible. Reinfection will occur if stressors persist. In salmonids, bacterial gill disease usually occurs in the spring and summer. In severe cases, cumulative mortality can exceed 50%.

D. Disease Signs

Clinical signs include lethargy, loss of appetite, increased gill activity, extended gill opercules, and fusion of gills filaments.

Histologically, a proliferative hyperplasia of the gill lamellae epithelium is observed. As the disease progresses, the proliferation of the epithelium causes a clubbing and fusion of the gill lamellae. Ruthenium red stain of the gill tissues reveals of bacterial cells adhered to the epithelium (Kudo and Kimura 1983a). (See Figure 1.)

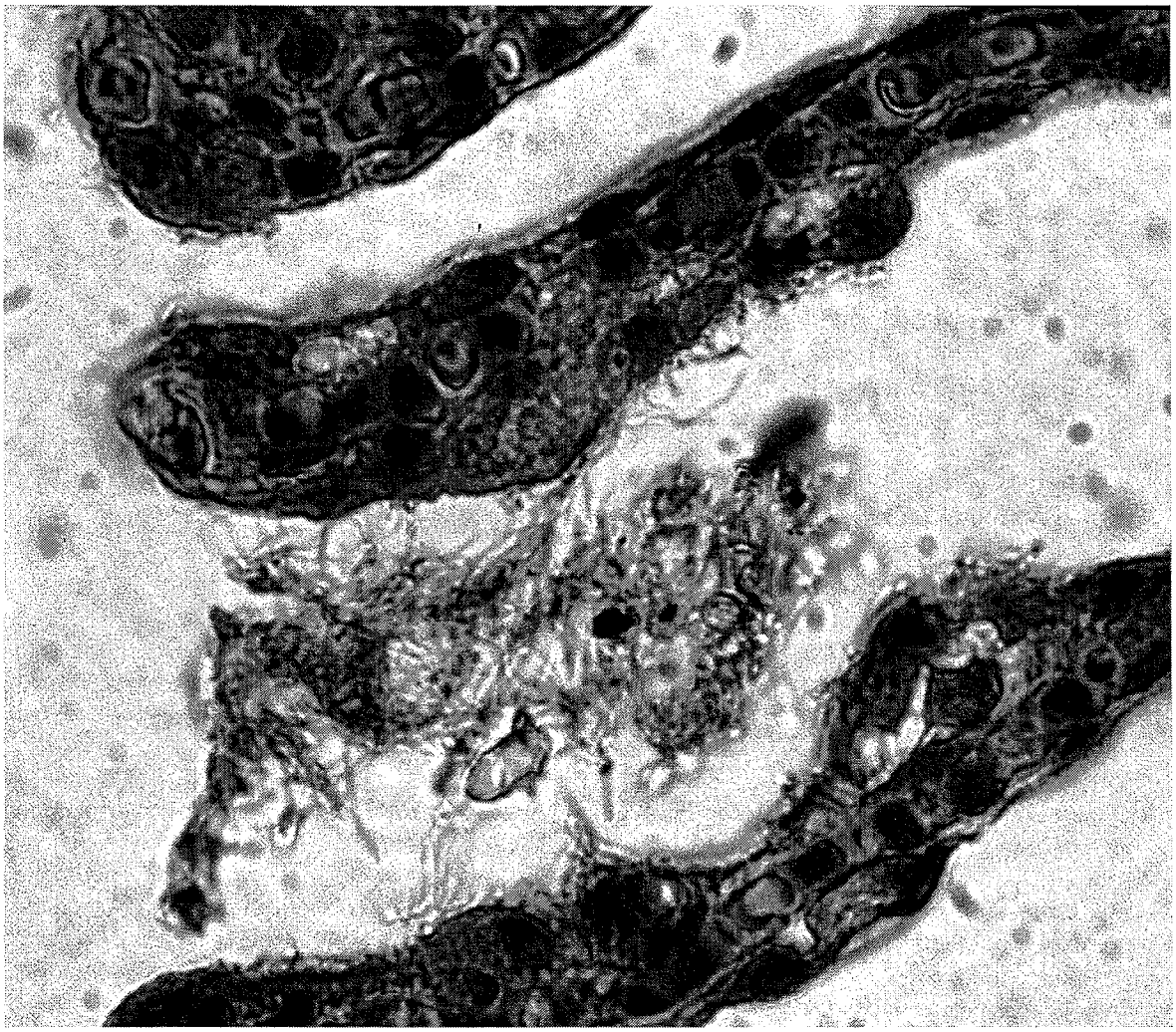

Figure 1. H&E section of gill from a rainbow trout showing large mats of flavobacterium causing bacterial gill disease. Photo courtesy of Dr. Chris Wilson.

E. Disease Diagnostic Procedures

Diagnosis is based on clinical signs along with examination of wet mounts of the gill lamellae for gill hyperplasia and the presence of bacteria. Enhanced observation of wet mount of gill filaments can be seen with phase contrast microscopy. Gram stain preparations of gill imprints can also be useful in diagnosis.

An FAT (Huh and Wakabayashi 1987) and ELISA (MacPhee et al. 1995b) test have been developed for *Flavobacterium branchiphilum* but are not in widespread use.

Diagnosis can also be based on histological examination of tissue sections stained with hematoxylin and eosin. Proliferation of the gill epithelium, and clubbing and fusion of the gill lamellae are diagnostic features. Bacteria can also be seen in tissue sections.

F. Procedures for Detecting Subclinical Infection

No procedures have been reported.

G. Procedures for Determining Prior Exposure to Etiological Agent

No procedures have been reported.

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Fish can be preserved in 10% neutral buffered formalin or Bouin's fixative for later histological examination.

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1.5 Columnaris Disease

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A. Name of Disease and Etiological Agent

Columnaris disease is caused by *Flavobacterium columnare.*

B. Known Geographical Range and Host Species of the Disease

1. **Geographical Range**
   Probably worldwide.

2. **Host Species**
   All freshwater fishes are considered susceptible.

C. Epizootiology

The disease affects fish of all ages and is favored by warm water conditions (14°C). The severity of columnaris is also affected by other environmental factors; for example, the impact of the disease may increase under conditions of low dissolved oxygen or high concentrations of ammonia. Mortality rates can be extremely high, with 60 to 90% mortality common. The precise reservoirs of the pathogen are unclear, but it may occur naturally in the aquatic environment. Large numbers of *Flavobacterium columnare* cells have been isolated from water during epizootics and good survival of the bacterium occurs over a wide range of water pH and hardness.

D. Disease Signs

When highly virulent strains of the bacterium are involved, the fish may die without any gross clinical signs but the pathogen is recoverable from the gills. With strains of lower virulence, external lesions of some diagnostic value are produced (internally, gross lesions are usually absent even though the pathogen may be present). External lesions may occur on the body surface, on the gills, or on both.

On scaled fish, lesions occur initially as greyish-white cutaneous foci on the fins, head, and trunk (Figure 1). The foci may enlarge to be several centimeters in diameter, and skin in the affected area may

be eroded, resulting in shallow ulcers (Figure 2). On the gills, the lesions appear to radiate from a focal point; the affected gill tissue becomes bleached and necrotic, but fusion of the lamellae does not occur. (Figure 3 and Figure 4). Often, the pathogen's yellow-pigmented cells may be present in large enough numbers to color the lesions yellow or orange (Figure 5). On scaleless fish, the center of the lesion appears to be a dark blue area covered by a milky veil with a defined red tinge (due to a hyperemia) around the margin. Sometimes called saddleback, these lesions resemble those caused by a fungus (Figure 6). The yellow pigmentation may also be seen around the edge of the lesion. In aquarium fish species, necrotizing stomatitis is common. This condition is popularly called cotton-wool mouth, and fungi are frequent secondary invaders. The infection may involve the opercula, teeth, maxillae, mandibles, and the spongy bones of the head.

Figure 1. Focal skin lesion caused by columnaris (picture by Andy Goodwin).

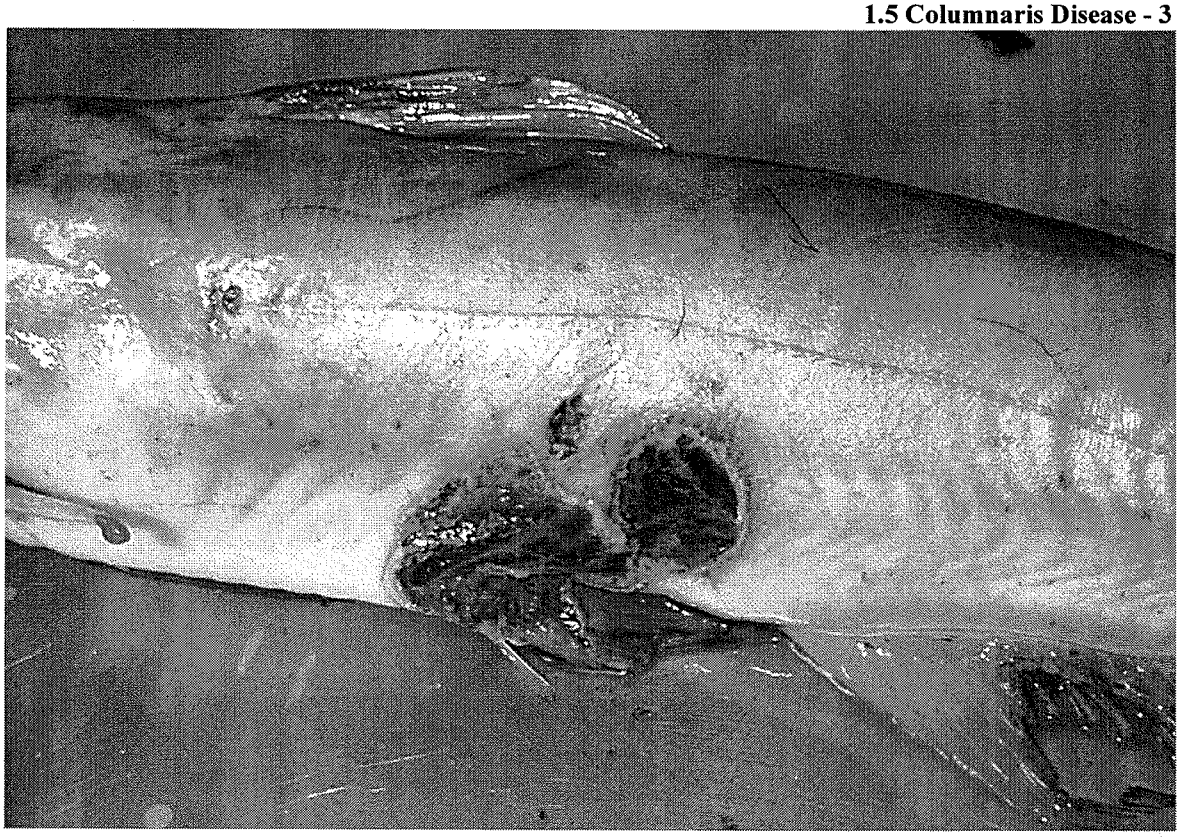

Figure 2. Shallow ulcers in channel catfish skin (picture by Andy Goodwin).

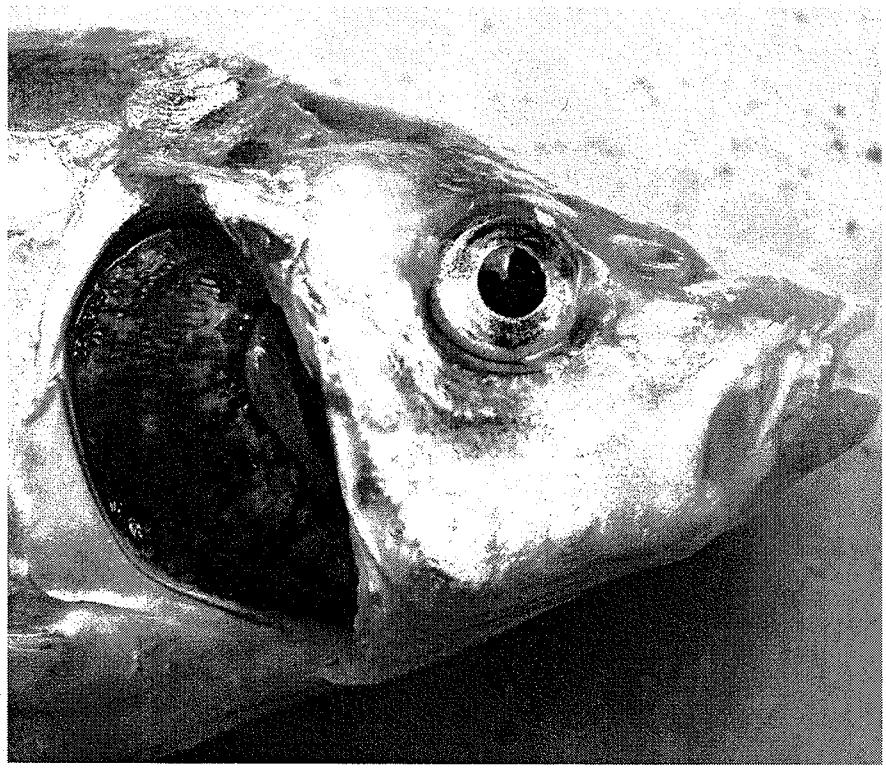

Figure 3. Columnaris gill lesions on a koi (picture by Andy Goodwin).

Figure 4. Columnaris gill lesions on a channel catfish (picture by Andy Goodwin).

Figure 5. Skin lesions on channel catfish. Sufficient columnaris are present to color the lesions yellow (picture by Andy Goodwin).

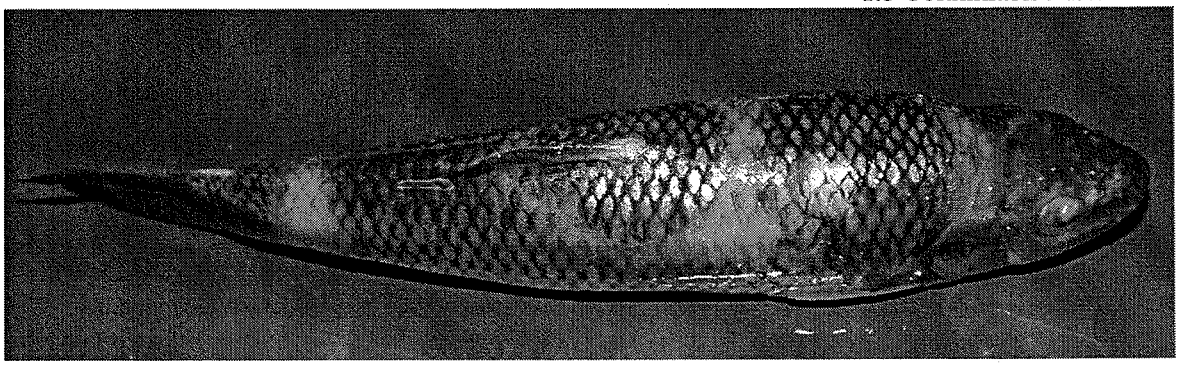

Figure 6. A bluehead chub with multiple saddleback lesions (picture by Andy Goodwin).

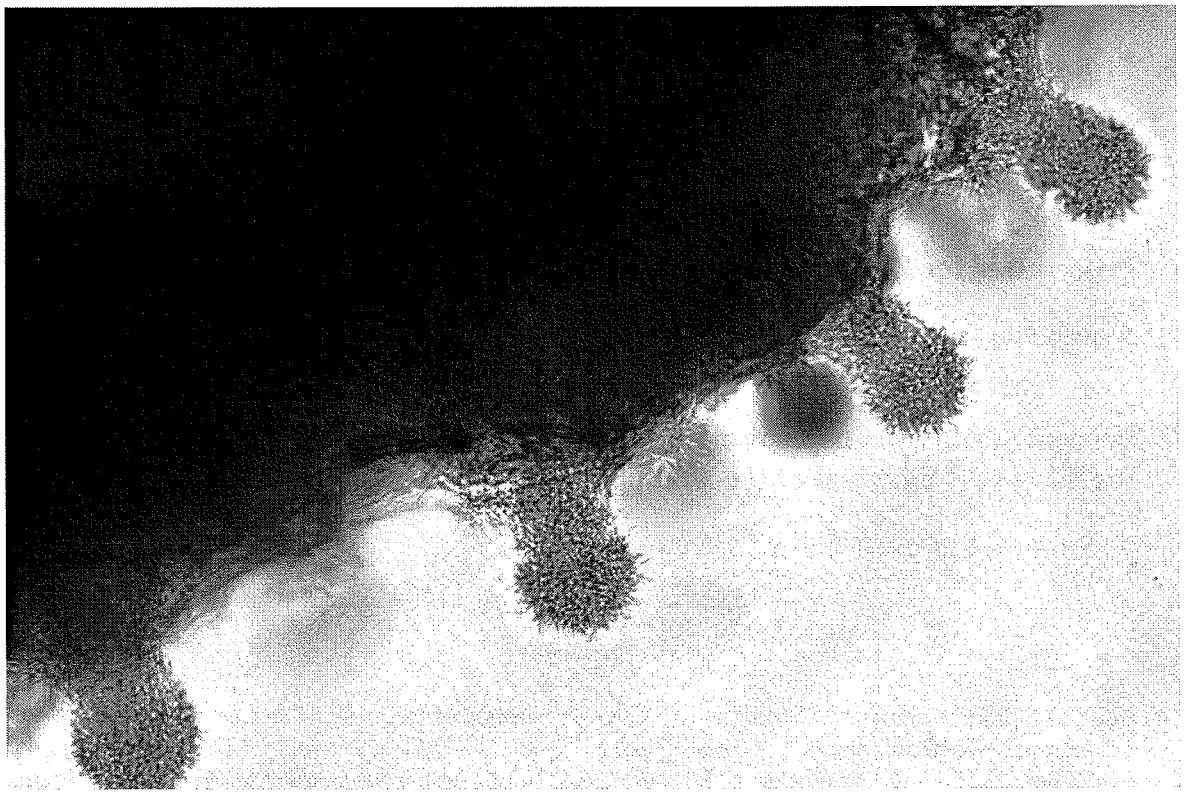

Figure 7. "Hay stacks" of *Flavobacterium columnare* on the gills of a koi(picture by Andy Goodwin).

E. Disease Diagnostic Procedures

1. **Presumptive Diagnosis**
   The following criteria provide a basis for presumptive diagnosis: observation of characteristic clinical signs and the presence of long, thin (5 to 17 x 0.7 um) gram-negative rods in lesions; dry rhizoid, yellowish colonies are produced on cytophaga agar (Anacker and Ordal 1959) after three days incubation at 20°C; cells are motile by gliding or flexing, but lack flagella. The tendency of the columnaris bacterium to form mounds (haystacks) or columns, as detected in wet mounts of diseased tissue, also aids diagnosis (Figure 7). The selective medium developed by Hsu and Shotts (Bullock et al. 1986) enhances isolation of *Flavobacterium columnare* from clinical specimens.

2. Confirmatory Diagnosis

a. Identification of *F. columnare* isolates may be accomplished by screening for: adherence of colonies to agar, colorshift in colonies from yellow to pink in presence of 3% sodium hydroxide, and production of chondroitinase and gelatinase. A PCR procedure is also available.

b. If the diagnostic antiserum is unavailable, an alternate confirmatory procedure is to show that the isolate is identical in its morphological, cultural, and biochemical features with *Flavobacterium columnare*. Descriptions of *Flavobacterium columnare* are provided in several of the papers listed in the references.

F. Procedures for Detecting Subclinical Infections

Because *Flavobacterium columnare* is considered ubiquitous in fresh water, a suitably sensitive detection procedure has not been developed.

G. Procedures for Determining Prior Exposure to the Etiological Agent.

No procedures have been reported.

H. Procedures for Transportation and storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent.

See Section 1, 1.1 General Procedures for Bacteriology.

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FISH DISEASE

Diagnosis and Treatment

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CHAPTER 4

The Clinical Work-Up

CASE SUBMISSIONS

Submissions to the Clinic

The basic steps that should be followed in the clinical work-up of a fish disease case are illustrated in Fig. I-1. If fish are submitted to the clinic, virtually all procedures can be handled on an outpatient basis, eliminating the need to keep fish overnight. Most cases will be initiated by a telephone call from an owner who is having a problem. The owner should be asked to bring in one to several representative fish for examination. It is important to determine whether the owner is amenable to the euthanization of any fish for the determination of a diagnosis. Hobbyists who are breeders are usually willing to sacrifice some fish, unless the fish are rare or expensive brood stock. While a complete postmortem examination is superior to performing only biopsies, this will not be possible in many pet fish cases; this can usually be discerned during the conversation with the owner.

If the client is submitting the fish to the clinic, the owner should be advised to bring both the fish and a water sample in *separate* clean containers. The best containers are a clean plastic bucket (never exposed to soap or other toxic chemical), plastic-lined cooler, styrofoam cooler, or a plastic aquarium bag. However, a ***well-washed and rinsed*** glass food container is also acceptable. Half a liter (one pint) of water is adequate for core water quality analysis.

To transport the fish (assuming the trip to the clinic will be less than 30 minutes), a good rule of thumb is to have about one liter of water for every one centimeter of fish (or one-half gallon of water for every 1 inch of fish) to be transported. Much higher densities can be used if supplemental oxygenation is provided. It is best to place the container of fish in a cooler to prevent temperature shock. Fish may also be transported directly in the cooler. For longer journeys, it is best to provide supplemental oxygen during transport. Oxygen cylinders or portable aerators (Baitsaver or equivalent) can be used to provide oxygenation. Alternatively, small fish can be shipped in a sealed plastic aquarium bag that has an oxygen-enriched atmosphere. The *Fish Disease Diagnosis Form (APPENDIX I)* provides details on various methods of shipment.

The ability to diagnose a problem is directly related to the quality of the samples submitted. Live fish that show typical clinical signs of the problem provide the best samples. Preserved material is least useful for most, but not all, diagnoses. Different methods of tissue storage are more useful for certain problems. Water samples also have a finite storage time (Table I-3).

Commercial Producers

While most individual pet fish cases are best submitted directly to the clinic, it is often important to visit the facilities of commercial growers, such as pet fish breeders, retailers, wholesalers, or commercial food fish producers. A visit allows a more thorough evaluation of the facilities and management, which are often the root cause of a disease complaint. The procedures used for diagnostic work-up are the same as for individual aquarium fish (see Fig. I-1).

Since more fish are usually involved in commercial producers' cases, more fish can be examined, which strengthens the diagnosis. Generally, four to six fish should be examined during epidemics. Live fish should be used whenever possible. The only exception is when all of the live fish appear healthy; in this case, the freshest dead fish should also be examined.

If fish are being certified for presence or absence of certain diseases, the number examined depends on the total population size, the prevalence of the disease to be surveyed, and the level of confidence desired (Sims & Schill, 1984). Amos (1985) and Thoesen (1994) provide detailed methods for certifying fish to be free of specific diseases.

WATER QUALITY ANALYSIS

Core Water Quality Parameters

Core water quality parameters are tests that should be run when any fish disease case is submitted. They include ammonia, nitrite, and pH (and salinity in a marine or brackish water system). Oxygen and temperature are also part of this core list but should be measured on site (i.e.,

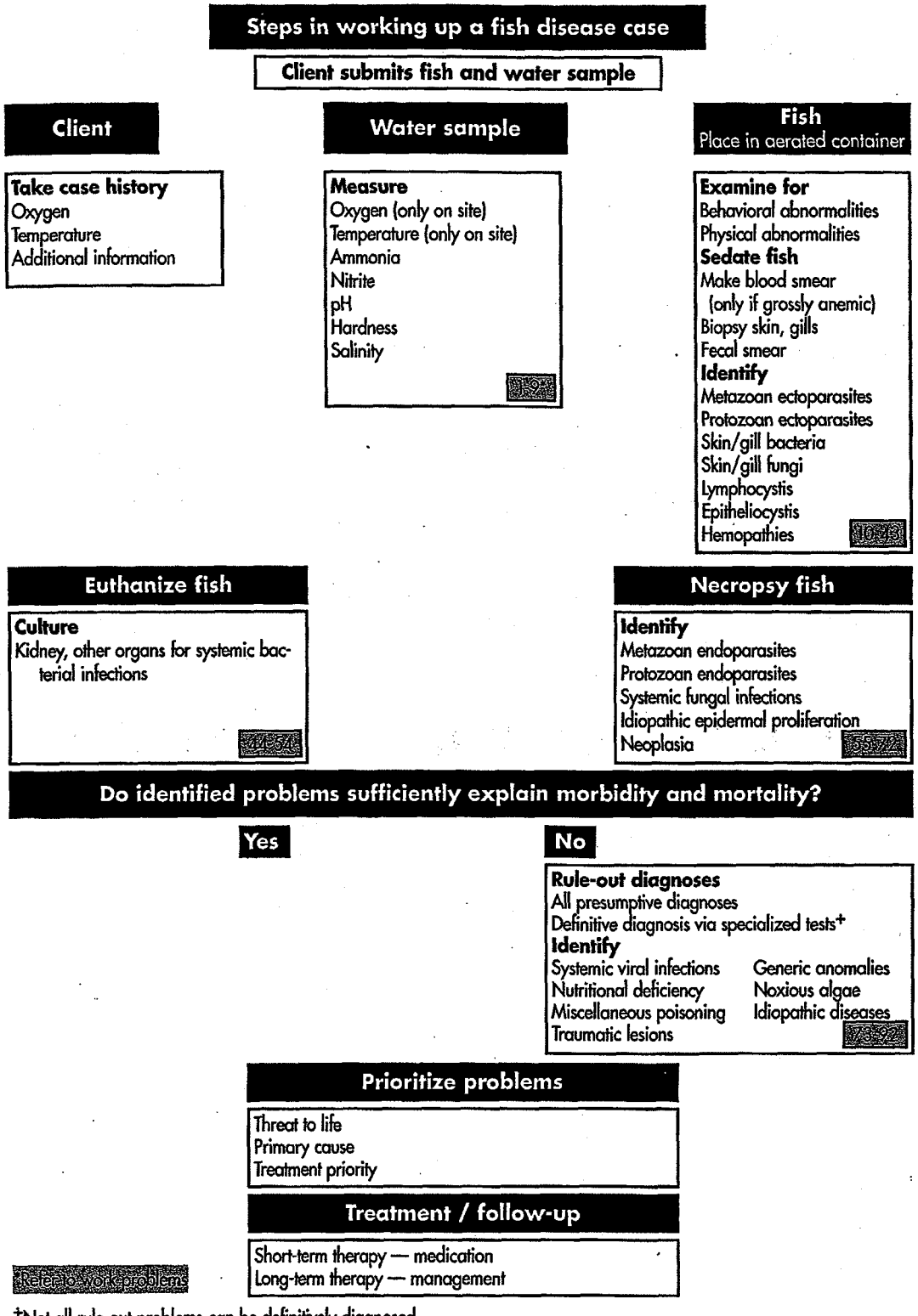

Fig. I-1 Steps in working up a fish disease case.

Table I-3 Recommended sampling containers and storage procedures for water samples.

Variable	Container	Volume (ml)	Handling procedure	Analyze within
All	Clean or new			ASAP
Oxygen	Glass stoppered glass	300	Fill totally, 4° in dark	6 hours
Temperature	N/A	N/A	N/A	None; must do on site
pH	Polyethylene	100	4° C in dark	6 hours
Ammonia; nitrate; nitrite	Polyethylene or glass (NOT HNO_3-washed)	500	Acidify with 1 ml concentrated H_2SO_4/L (to pH <2.0); store on ice or freeze 4° C	24 hours 24 hours
Metals	Polyethylene, HNO_3-washed	500	Acidify with analytical HNO_3 to pH <2.0; freeze if analysis delayed	24 hours
Pesticides, other organochemicals	Glass- or Teflon-stoppered glass, hexane-washed (no plastics)	500	Fill totally	24 hours
Solids (dissolved; suspended; settleable)	Glass or plastic	500	4° C	
Cyanides	Glass or plastic	100	Add 0.2 ml of 10 M NaOH, to pH 12	24 hours
Algae	Glass or plastic	100	Fresh chilled, or add Lugol's iodine to color of weak tea or add 10% formalin 1 : 1	24 hours
Summary (agents unknown)	Polyethylene; HNO_3-washed	500	Add HNO_3 to pH <2.0	24 hours
	Glass or plastic; (2 samples)	500	Freeze one	24 hours
	Glass; hexane-washed	500	Fill totally	24 hours
	Glass; hexane-washed	500	Fill totally, 4° C in dark	6 hours

From Langdon, 1988; Hill, 1983; Boyd, 1979. Suggested time intervals should be considered liberal estimates. Samples may be less stable under some conditions.
HNO_3 = nitric acid; H_2SO_4 = sulfuric acid; NaOH = sodium hydroxide.

at the pond, aquarium) to be accurate; this can be done only if the clinician visits the site. Otherwise, oxygen and temperature must be assessed from the history (i.e., the client has measured the oxygen or temperature; or, a problem with oxygen or temperature is discerned from the client interview).

While it is not part of the core list, it is often advisable to measure alkalinity and hardness in commercial ponds and nitrate in aquaria (especially marine aquaria). Chloride should also be measured in commercial ponds when nitrite levels are high (see *PROBLEM 5*).

Special (Noncore) Water Quality Sampling

Many other water quality changes besides the core list can affect fish health (see *PROBLEMS 80* through *86*). While not routinely measured, some cases may warrant examining these other factors (see *RULE-OUT DIAGNOSES* and Fig. I-1). Specific recommendations for sample collection vary with the type of substance being measured and with how quickly the sample can be submitted (i.e., will preservative be added?). Also, different types of samples need to be collected in different types of sample containers (plastic, glass). After determining that certain measurements should be taken, the clinician should contact the laboratory where the samples are to be submitted to obtain specific information on methods of collection. The American Public Health Association (A.P.H.A., 1992) also provides extensive details on water sampling.

Water Quality Testing

Many manufacturers produce simple test kits for measuring core water quality parameters and other water quality variables. Most tests are based on adding a known amount of the water sample to a vial and then adding chemicals, which react with the substance to be measured, producing a colored reaction. The amount of substance present is proportional to the intensity of the color change. Most tests take less than 5 minutes to run. It is important to realize that special procedures are sometimes required to test substances in seawater; thus, while most kits for measurements in seawater are also usable for freshwater samples, the converse is not always true.

The accuracy of commercial water test kits is related to the cost of the kit. Inexpensive kits that use a color chart for measurement are available from aquarium wholesalers or retailers (e.g., Marine Enterprises). These water test kits are only semiquantitative but give a general indication of water quality and are often sensitive enough to diagnose most water quality problems encountered in routine clinical cases. More expensive kits designed specifically for water quality testing on commercial farms (e.g., FF-1A Kit [approximately $90]; FF-2 Kit [approximately $350];

Hach Company) are more accurate and acceptable for all routine diagnostic procedures; these also have the advantage of combining most routine tests into one kit. Even more sophisticated colorimetric kits use a spectrophotometer for measurements (e.g., Hach DREL 2000, approximately $3000) and are usually accurate to within 20% of the so-called *standard methods* (Boyd, 1979).

The most accurate methods for water quality analyses are the standard methods. In the United States, most standard methods are developed and sanctioned under the auspices of either the American Public Health Association (A.P.H.A., 1992) or the United States Environmental Protection Agency (U.S.E.P.A., 1979). Standard methods of analytical accuracy are not needed for clinical diagnoses, unless a particular case may eventually involve litigation or is involved in certain research protocols. Samples taken for regulatory compliance monitoring or collected as evidence during enforcement investigations must also conform to well-defined procedures regarding sample handling, shipment, and chain-of-custody documentation. The clinician should refer to EPA guidelines or contact the appropriate environmental agency (e.g., U.S.E.P.A or regional or state environmental agency) for assistance in collecting such samples.

If frequent visits to culture facilities are anticipated, it is also advisable to purchase a dissolved oxygen meter (e.g., YSI about $600). Electronic probes are also available for measuring temperature, pH, nitrite, chloride, and conductivity (salinity). The major advantage of electronic probes is that measurements can be taken quickly and accurately. However, probes are expensive, must be calibrated regularly, and are subject to failure if they are not maintained properly. It is also desirable that probes withstand disinfection, reducing the potential transmission of disease. For example, YSI dissolved oxygen probes can be left in disinfectant indefinitely, including 70% ethanol, povidone-iodine, quaternary ammonium, or just about any chemical that does not damage the housing (e.g., does not chemically react with the plastic housing; the probe itself is inert, being Teflon). Details of various water sampling devices are described with specific water quality problems.

Water Samples Submitted to the Clinic

The water sample should be immediately examined for core water quality parameters because changes can occur within a short time after collection (see Table I-3). If it cannot be examined immediately but will be examined within 1 hour, it should be left at room temperature. If it will not be examined for over an hour, it should be refrigerated but should be tested for ammonia, nitrite, and pH within 24 hours. The water should be allowed to come back to room temperature before doing any measurements.

Water Sampling on Site

Water samples may vary tremendously from one part of a culture system to the other. For example, oxygen and pH are highest, while carbon dioxide and ammonia are lowest, at the inflow of a flow-through system. The opposite is true at the outflow. Thus, flow-through systems should be sampled for oxygen, pH, and ammonia at both the inflow and the effluent.

Ponds should be sampled for dissolved oxygen and temperature at both the windward and leeward sides to account for wind-induced mixing (Boyd, 1990). Samples should be taken at 0.5 to 1.0 m (1.5 to 3 feet) in waters less than 2.0 m (6 feet) deep. Both surface and water samples should be taken to assess variability. Different bodies of water can have markedly different water quality characteristics, even with identical stocking densities, feeding rates, etc. (Noga & Francis-Floyd, 1991). Thus, each system should be treated as an individual unit in terms of water quality sampling.

TAKING THE HISTORY

When ready to see the client, a thorough history should be taken (see *Fish Disease Diagnosis Form, APPENDIX I*) (Stoskopf, 1988). It can be useful to try to determine whether the problem is acute or chronic, since this can help to eliminate some differentials (Fig. I-2). Acute problems are typically those that have developed within a matter of only a few days and have resulted in considerable morbidity and/or mortality within that time. Conversely, chronic problems typically develop over several weeks or more and may only result in an occasional mortality. Also, such fish are often in poor condition and may be anorexic.

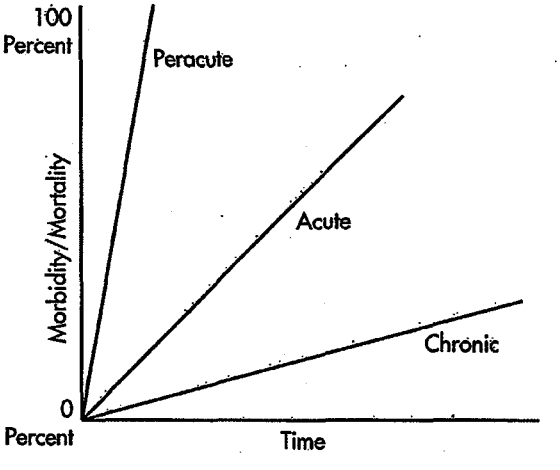

Fig. I-2 Typical morbidity and/or mortality rates with peracute, acute, or chronic disease.

Important questions to ask include the following:

- How long has the culture system (aquarium, pond, etc.) held fish?
- Are all fish affected?
- If not, which are not?
- Do the fish display any behavioral signs, such as *flashing* (rubbing against objects suggesting skin problems) or *piping* (staying near the air-water interface to obtain more oxygen, suggesting gill problems)? Low oxygen is common; unfortunately, oxygen can only be accurately measured on site (i.e., at the pond), so the history may be crucial to assessment.

It is often best to ask the client to describe the usual routine for feeding, water changes, and other management procedures to discern an accurate history. It is also important to determine what prior treatments, including medications, have been given.

THE PHYSICAL EXAM

When a client submits live fish to the clinic, aeration should be immediately placed into the container that holds the fish. Once a thorough history has been taken, the fish should be closely examined for behavioral abnormalities (e.g., increased respiration, suggesting possible gill problems or nitrite toxicity) (Francis-Floyd, 1988) and external lesions (e.g., ulcers, masses, *cloudiness* of the skin caused by increased mucus production, erosion or irregularities of the fins, etc.). Examining gills is most easily done when taking biopsies (see *GILL BIOPSIES*).

Color Change as an Indicator of Fish Health

The melanin pigmentation in fish's skin is under neuroendocrine control and is thus affected by hormones, such as epinephrine. When fish are sick, maintenance of a normal pigmentation pattern presumably takes less precedence than homeostasis of more vital body functions. Thus, sick fish are often abnormally colored, compared with the healthier tankmates. This is a common response of salmonids to disease, with sick fish being typically darker than normal. A color change can also be caused by blindness, which eliminates the normal visual cues that are needed to maintain a normal color pattern in daylight (Fig. I-3, *A*). Fish in breeding condition often have more brilliant colors than nonbreeding fish (Axelrod et al., 1980). Since the chemical signals that control pigmentation are transmitted via the nerves, peripheral nerve damage, such as from vertebral instability, can cause a focal change in pigmentation pattern (see *PROBLEM 64*). Focal color change can also be caused by local tissue irritation/damage, such as parasite feeding, chronic wounds, or healing wounds, which cause a change in the pigment cell distribution at that site (see *PROBLEMS 53* and *55*).

Reddening of the body is usually caused by hemorrhage, which can result from systemic bacterial or viral infections (see *PROBLEMS 44* and *73*), or skin wounds. Loss of fin tissue most often results from poor water quality (see *PROBLEM 36*). Parasites may also incite a thickening of the skin, which leads to a whitish cast or white foci (see *PROBLEM 19*). See *EVALUATION OF SKIN BIOPSIES*, p. 21, for a further discussion of gross lesions affecting the skin. Observations of color pattern are best made while the fish is in its culture system, since the pattern can also be affected by acute stress (e.g., confinement).

Other Common Gross Signs of Disease

Sick fish often congregate together, separating themselves from their healthier cohorts (Fig. I-3, *A*). Weak fish in raceways or other systems with flowing water will often be found near the water outlet. Sick fish may also exhibit other behavioral signs, including staying near the surface of the water because of hypoxia (see *PROBLEM 1*), scraping the body because of parasite irritation, or showing various behavioral abnormalities because of nervous system involvement (see *PROBLEM 74*).

Another common clinical sign is abdominal swelling (Fig. I-3, *B*), which is most commonly caused by an infectious peritonitis (viral, bacterial, or parasitic) but can also be caused by a metabolic disturbance (e.g., renal failure), neoplasia, or obesity. This clinical sign is often referred to as *dropsy* in the aquarium literature. Abdominal swelling may also be a normal sign of sexual maturity in female fish that are ready to spawn.

Eye lesions, such as exophthalmos (Fig. I-3, *C*) are common in several infectious diseases, including several viral and bacterial infections. Unilateral lesions often indicate a possible traumatic cause, especially in large fish. Many nutritional deficiencies are also associated with ocular pathology.

Skeletal deformities (Fig. I-3, *D*), especially of the vertebral column, are also seen in several types of diseases, ranging from certain parasitic infections to nutritional deficiencies to toxicoses.

See *GILL BIOPSY PROCEDURE*, p. 23, and *COMMON LESIONS FOUND IN THE VISCERA*, p. 41, for other gross signs of disease.

PREPARING FISH FOR BIOPSY

Latex gloves should be worn when handling fish for disease diagnosis. Fish skin is not keratinized and thus is susceptible to iatrogenic damage. **(A dry paper towel should never be used to grab a fish for biopsy!)** Latex gloves are soft and slippery when wet, reducing possible skin damage and preventing the loss of surface-dwelling

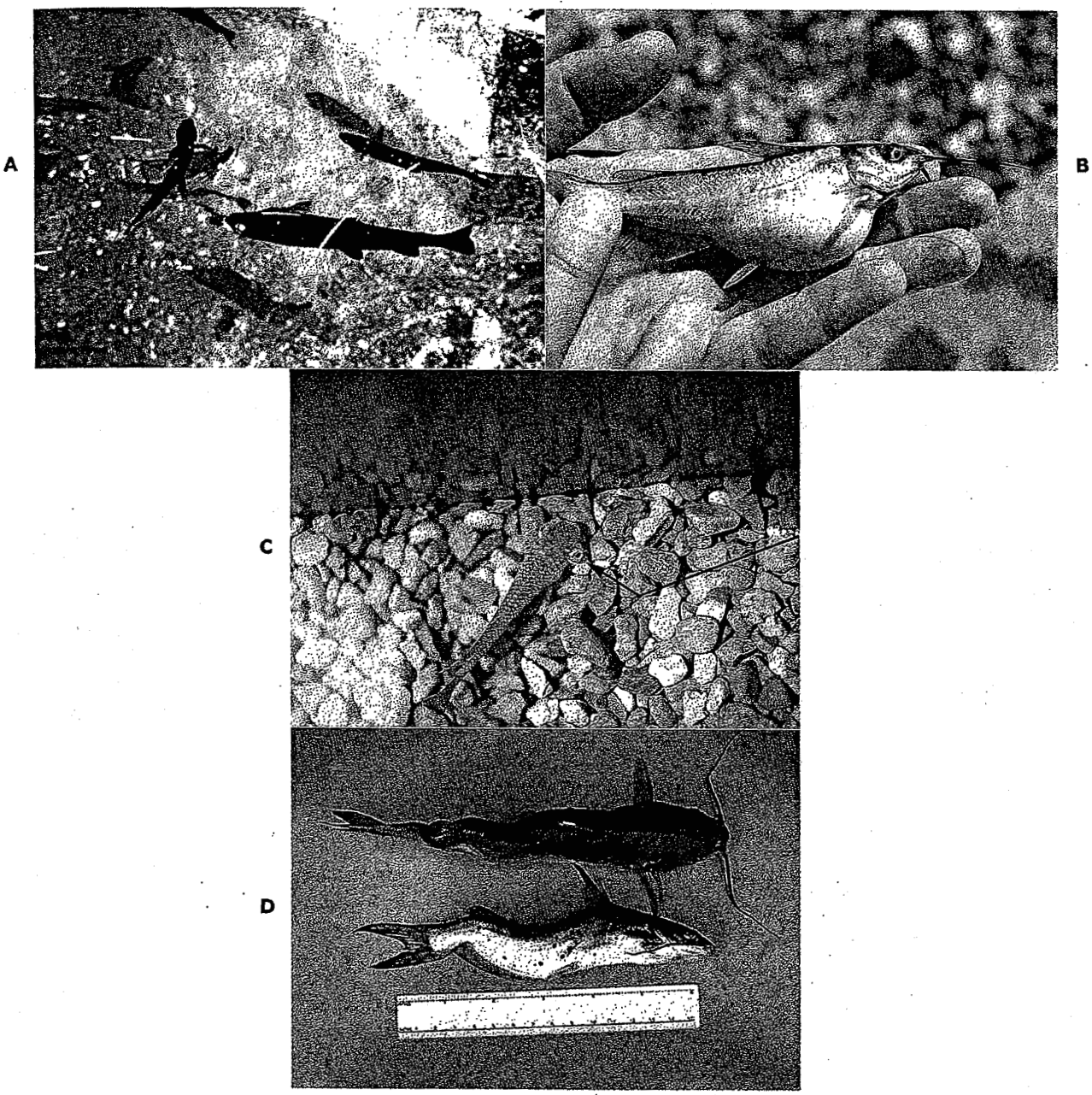

Fig. 1-3 Common gross signs of disease in fish. **A,** Salmonids congregating near the outlet screen of a raceway. In this case the dark color is caused by blindness. But, color change is a general indicator of ill health. The segregation of these fish away from the rest of the fish population is also characteristic of sick fish. **B,** Massive swelling in a channel catfish caused by fluid accumulation in the peritoneal cavity. **C,** Exophthalmos (*arrow*) in a killifish. **D,** Spinal curvature, including scoliosis (*lateral curvature*) and lordosis (*forward curvature*).

(*A* photograph courtesy of C.L. Davis, Foundation for Veterinary Pathology; *B* and *C* photographs courtesy of T. Wenzel; *D* photograph courtesy of A. Mitchell.)

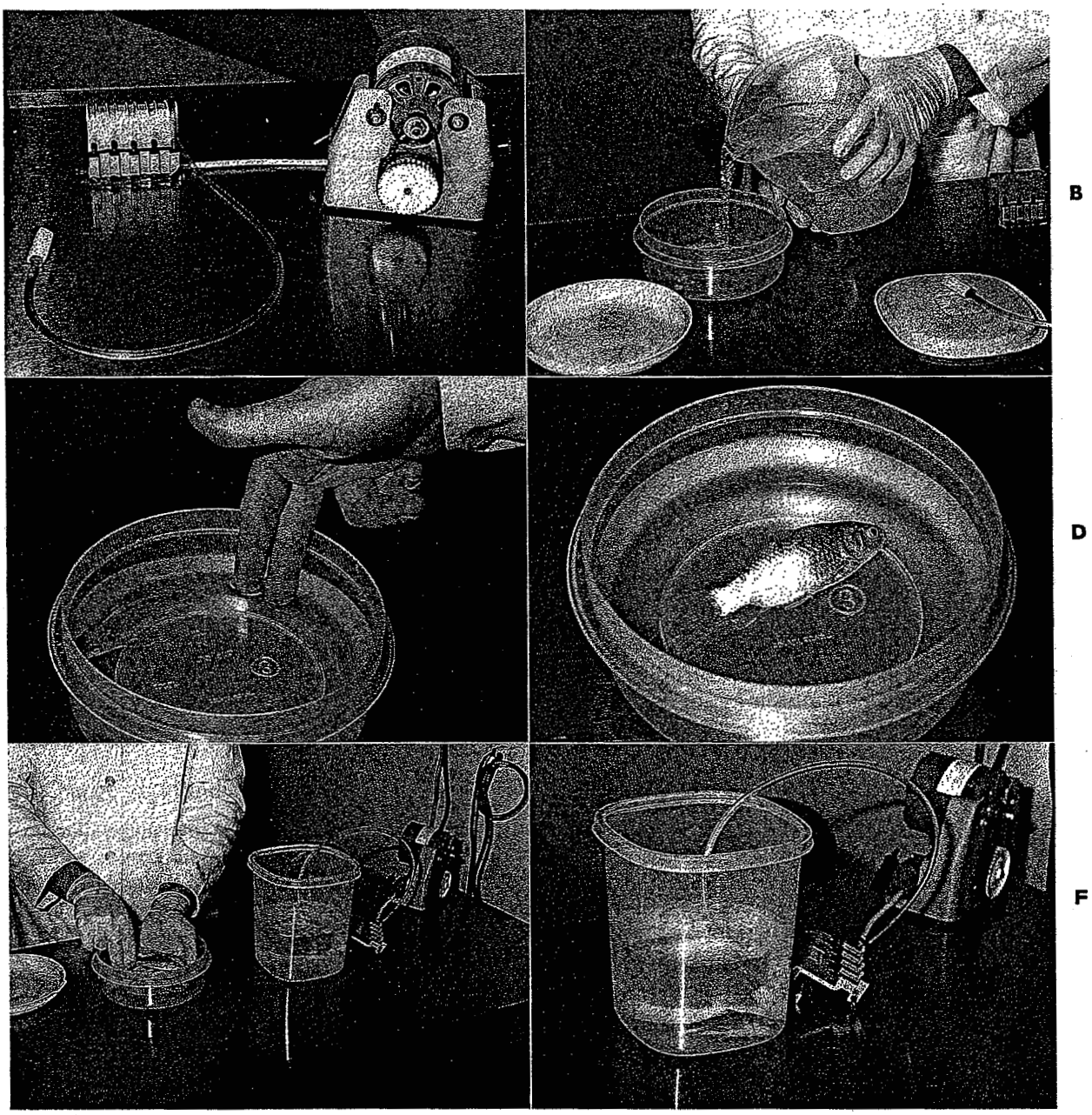

Fig. I-4 Method for sedating fish before a clinical procedure. A, A simple device for providing aeration during a clinical visit. An electrical air pump is attached to a gang valve having five outlets, so that up to five containers can be aerated at once. B, Adding some water from the container in which the fish was submitted to another container to be used for sedation. C, Mixing the tranquilizer/anesthetic before adding the fish. D, Fish is responding to sedation (losing balance). E, Fish is being removed for a clinical procedure. F, Fish placed in aerated water after completion of the clinical procedure.

parasites when handling the fish. Also, some zoonotic pathogens can be contracted by handling infected fish (see *PROBLEMS 45, 48, 49,* and *53*). Many disposable gloves are coated with talc, so gloved hands should be rinsed in water before handling the fish to prevent talc crystals from contaminating biopsies (see Fig. II-42, *C*).

After the visual examination, the skin and gills should be biopsied to look for pathogens. Skin biopsies usually can be taken from any fish larger than 25 mm (1 inch), and a gill biopsy can usually be taken from any fish larger than 50 mm (2 inches). These techniques are valuable because many of the diseases that affect fish are confined to the skin or gills.

Sedation / Anesthesia

The same drugs are used for both sedation and anesthesia in fish. The only difference between sedation and anesthesia is the dosage of drug and/or the length of time that the fish is exposed. Since these drugs are all administered through the water, the dosage is directly proportional to both the amount of drug in the water and how long the fish has been left in the solution.

For biopsy, a portion of the water used to transport the fish is placed into an aquarium bag or other suitable container and a small amount of anesthetic (and buffer, if necessary) is added (see the *PHARMACOPOEIA* for types of anesthetics available) (Fig. I-4, *A* through *F*). The fish is then placed in the anesthetic bath and watched carefully. The *PHARMACOPOEIA* provides a range of doses that have been used for various fish species. Response to a given dosage varies considerably, depending on fish species and environmental conditions. When these drugs are used on a fish species with unknown susceptibility, start with the lower recommended dose and gradually add more if needed, until the desired effect is reached.

Fish exhibit planes of anesthesia that are similar to mammals. The first stage is excitation; some fish, such as eels, struggle violently during this stage and may attempt to escape. The container that holds such fish should be well covered. After excitation the fish becomes depressed (less response to touch), loses equilibrium (lies increasingly on its side), and respiration slows (gilling, the opening and closing of the gill covers, becomes slower and weaker). If the fish is left in the anesthetic bath long enough, breathing will stop. Fish should not be left in anesthetic long enough to stop breathing; however, many fish will recover even after breathing has stopped for several minutes.

If the proper amount of anesthetic is added, the fish should be immobilized in less than 5 minutes. If the fish remains alert after this time, a bit more anesthetic should be added. Once the fish has ceased to struggle and can be handled, a fin clip should be taken with fine forceps and a skin scrape should be taken with a scalpel. These biopsies should be placed immediately on a slide with a drop of aquarium water, a coverslip should be added, and then the specimen can be examined. A gill biopsy should then be taken, using fine scissors.

Anesthesia often causes involuntary defecation, allowing the collection of a fecal sample (see *FECAL EXAM*). Sedation or anesthesia may also cause some loosely attached ectoparasites, such as monogeneans or leeches, to detach from the fish (Noga et al., 1990; Svendsen & Haug, 1991). It could interfere with diagnosis of these problems by biasing the number of organisms observed on wet mounts. However, the importance of parasite narcotization on making a clinical diagnosis has not been studied. With practice, many fish can be biopsied without sedation. If the fish can be euthanized, pithing or cervical severance can be used for immobilization rather than chemical overdose (see *PHARMACOPOEIA*).

USING THE MICROSCOPE

Next to water testing, examining tissues by wet mount is the most informative technique in fish disease diagnosis. In fact, the majority of fish disease cases can be diagnosed by using just the water quality tests outlined and by an examination of skin and gill wet mounts.

The microscope used for diagnosis should have a range of objectives, including at least 10X, 40X (low and high dry) and 100X (oil immersion, high power). A low-power (4X) objective is also useful for rapidly scanning a sample. Close down the iris diaphragm to exclude much of the light and increase contrast. When wet mounts are examined, it is important to determine the size of various objects in the microscope's field because the proper identification of a parasite or other organism is much easier when its size is known.

The most accurate way to measure an object's size is to use an ocular micrometer. This micrometer is placed into the eyepiece of the microscope and can then be superimposed over the organism in question to measure its size. Another way to measure the size of an object is to compare it to the size of a red blood cell (RBC) in the same field. Fish RBCs usually range from about 6 to 9 µm on the long axis. They can be identified on a wet mount by their platter-shaped or fried-egg appearance (see Fig. I-11). Because the RBCs are fairly consistent in size, they can be used to estimate the dimensions of an object. Latex beads can also be used for size estimation.

BIOPSY PROCEDURES: PREPARING SLIDES

Immediately before performing any biopsies, a drop of water (seawater, if it is a marine fish) is added to a slide for every biopsy that is to be performed on the fish. Water from the container that holds the fish can be used (Fig. I-

5, *A*). One of the quickest ways to transfer the water is to dip the tip of your finger into it and then touch your finger to the slide. This will leave a small drop of water on the slide. A pipet can also be used. The biopsy should be placed immediately in the water drop to prevent any organisms in the sample from drying out and thus dying.

SKIN BIOPSY

Skin biopsy is the single most useful tool available for diagnosing diseases in fish because the skin is a primary target organ for a number of common infectious agents. The skin of fish has layers analogous to those present in mammals, including the hypodermis, dermis, basement membrane, epidermis, and cuticle (Fig. I-6, *B*). The dermis contains pigment cells and the scales, which are embedded in connective tissue and overlap one another like shingles on a roof. Some species, such as catfish, lack scales, while others, such as eels, have small scales. Covering the scales is the epidermis, a stratified squamous epithelium with goblet (mucus-producing) cells. The epidermis is covered by the cuticle, a thin layer of mucus that contains sloughed epithelial cells and many protective substances, such as antibody, lysozyme, and C-reactive protein (Alexander & Ingram, 1992). In almost all fish, the epithelium is not keratinized, and living cells are present in all layers. This makes fish skin susceptible to both acute and chronic injury.

The skin performs the following three functions in all fish: (1) it reduces drag by providing a smooth friction-free surface for locomotion; (2) it acts as a first line of defense against the invasion of infectious agents; and (3) it makes an impermeable barrier to the movement of fluids and salts. In some species (e.g., eels, catfish), it also acts as an accessory site for respiration. Its critical importance in maintaining internal homeostasis is a major reason why skin damage, exclusive of other organ involvement, can kill fish.

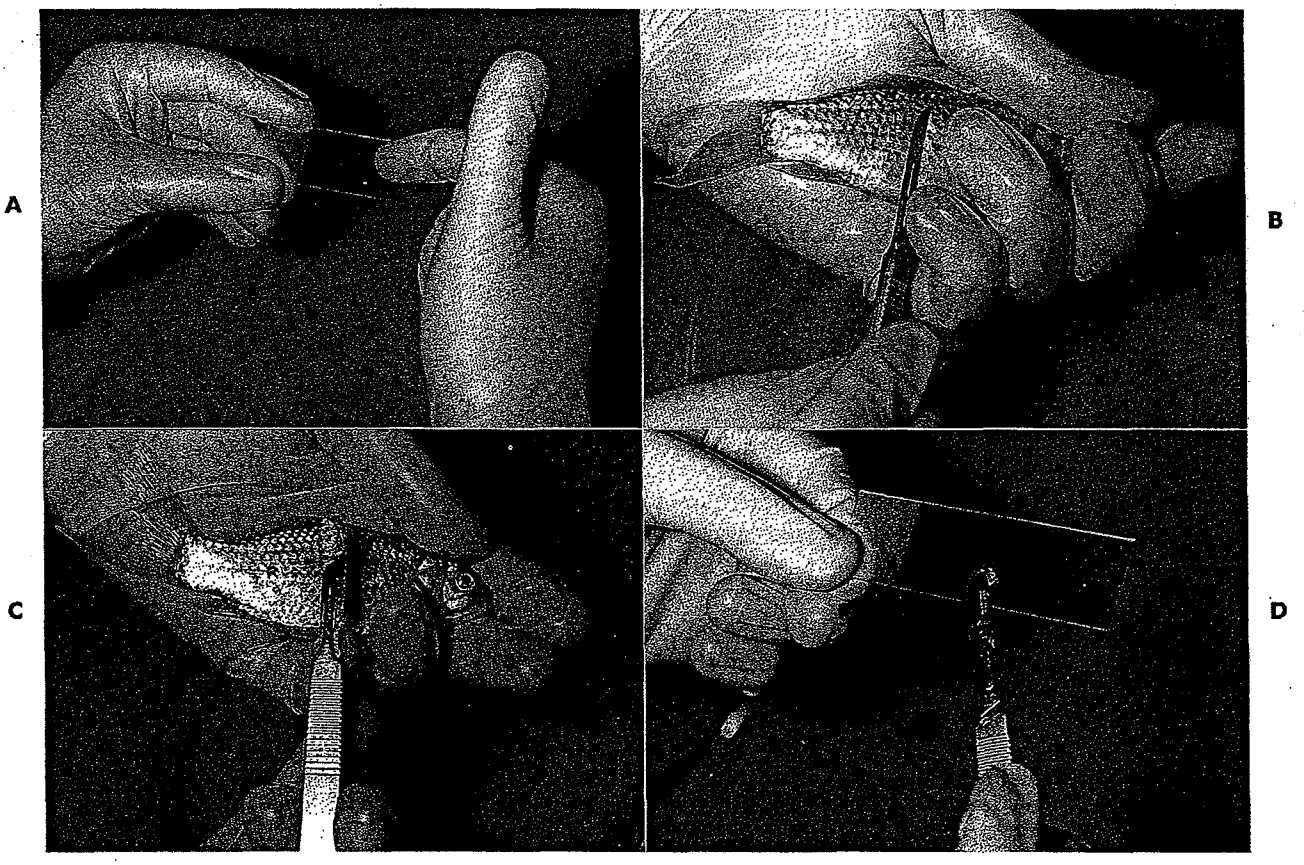

Fig. I-5 The skin scraping. **A,** Adding a drop of water to a slide before performing the biopsy. Dip a finger in water, and then touch the finger to the slide. **B,** Scraping the skin with a scalpel to obtain a biopsy sample. Note that the back side of the blade is used for scraping. Only a relatively small area (*dotted line*) should be scraped. **C,** Biopsy material on the scalpel blade. Note that scales (flat, refractile) have been included in the biopsy, indicating that the entire epithelial layer has been removed. **D,** Scraping the biopsy material onto the slide.

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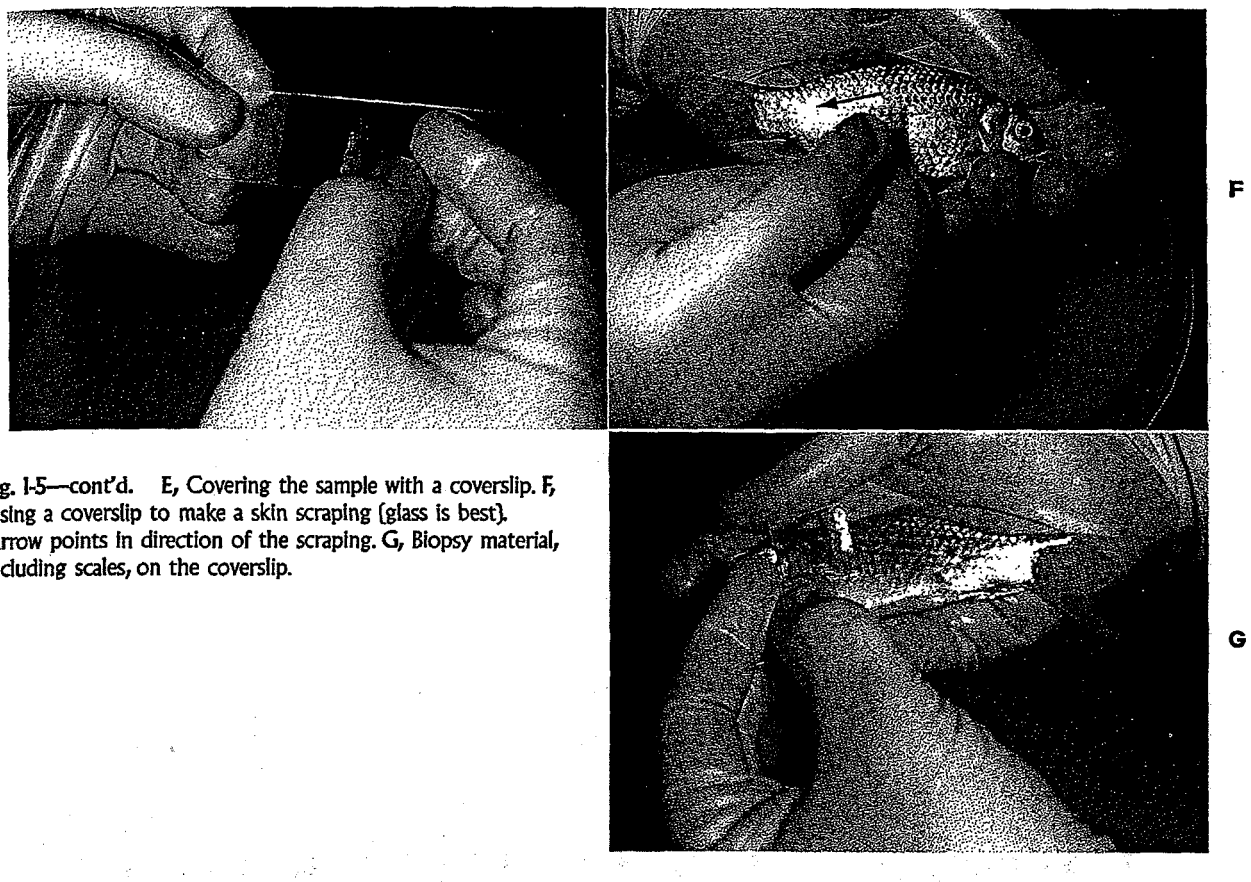

Fig. I-5—cont'd. E, Covering the sample with a coverslip. F, Using a coverslip to make a skin scraping (glass is best). Arrow points in direction of the scraping. G, Biopsy material, including scales, on the coverslip.

SKIN BIOPSY PROCEDURES: SCRAPING

Two major methods are used to obtain skin biopsies: skin scraping and fin clipping. Skin scraping is performed by taking a spatula or a scalpel and gently scraping along the side of the body or fins while the fish is adequately restrained (Fig. I-5, *B* through *G*). Lightly sedated fish can usually be prevented from struggling by enclosing the body with a loosely clasped hand. Avoid damaging the skin when performing any procedures by not exposing the fish to dry or rough surfaces. For example, fish should not be held with paper towels, even if the towels are moistened. This rough surface can easily remove the cuticle. Latex rubber gloves moistened with water are especially good for handling fish.

Only gentle pressure is necessary when taking a scraping because most pathogens are found near the surface. Much less pressure is required than that used in performing skin scrapings of mammals. Overzealous sampling does more harm than good. Even light scrapings usually remove the epidermis and dermis from small fish (see Figs. I-5, *C*, and I-6, *A*). Large areas of skin should not be scraped because the resulting open wound may become secondarily infected or cause serious fluid imbalance.

Scrapings should be taken where obvious lesions are present. The smaller wounds should be examined carefully since older lesions are often overgrown by opportunistic bacteria (e.g., *Aeromonas hydrophila*, see *PROBLEM 45*) or water molds (see *PROBLEM 33*). The leading edge of a lesion should always be examined because this area is most likely to harbor the inciting pathogen(s). To determine the initiating etiological agent may require sampling sites other than obvious lesions to discover which pathogens are present and also examining other fish in the same group. When pathogens are not detected by wet mount, bacterial culture of lesions is warranted.

The scraping should be immediately transferred to a glass slide, applying a drop of water (seawater, if a marine fish) and a plastic coverslip. Plastic coverslips are preferred to glass, since they are inexpensive and are less easily broken when crushing wet mounts from viscera. The wet mount should be examined immediately, since many parasites, especially the protozoa, will die soon after being removed from their hosts. Most parasites are difficult to identify when dead. It can be helpful to apply a drop of methylcellulose solution (Carolina Biological Supply Company) to slow the movement of protozoa, but this is almost never needed for identification of parasites.

Fungal hyphae, granulomas, and most protozoa are visible at low (40X to 100X) magnification under the microscope. The definitive identification of protozoa and bacteria usually requires high dry magnification (400X) and sometimes oil immersion (1000X).

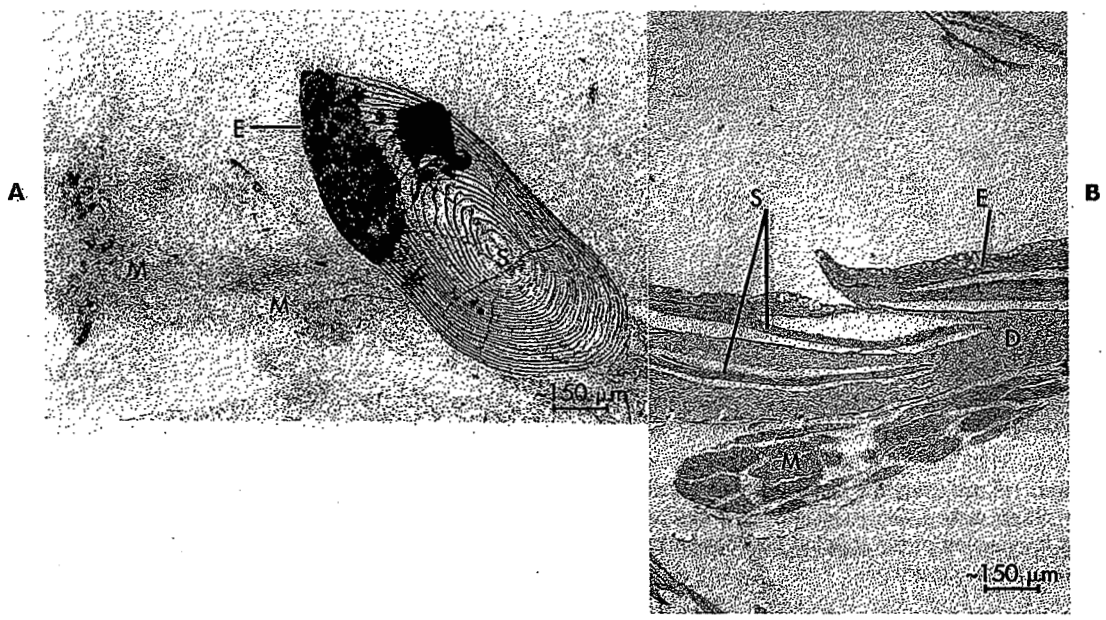

Fig. I-6 A, Low-power photomicrograph of a scraping from normal skin of a black-pigmented fish showing a scale (*S*), dark epithelium (*E*) covering the posterior part of the scale and mucus, and epithelium (*M*) scraped from the skin. B, Histological section of normal skin. The space between the scale and dermis is an artifact caused by shrinkage during histological preparation. *S* = scale; *E* = epithelium; *D* = dermis; *M* = muscle; hematoxylin and eosin.

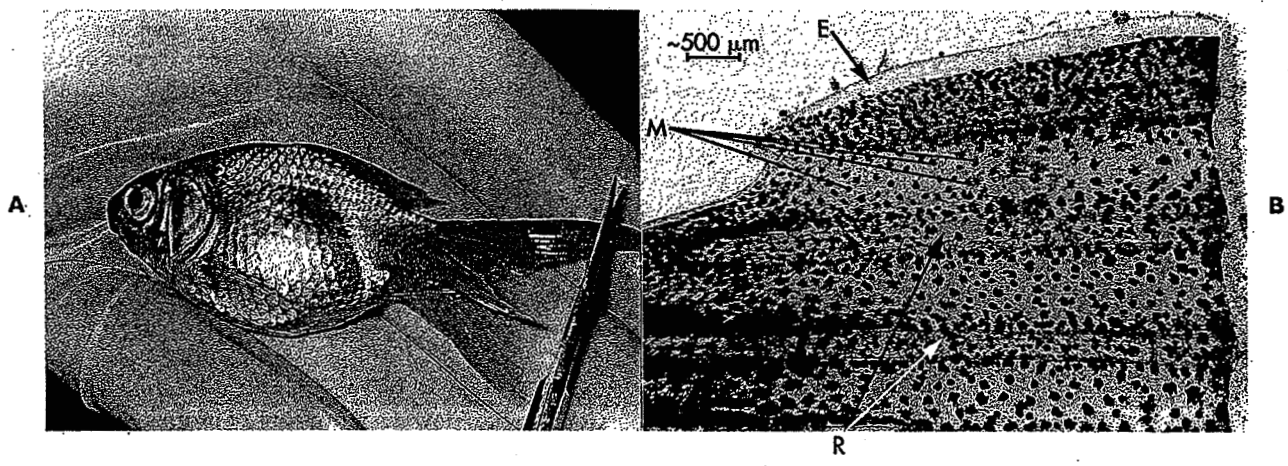

Fig. I-7 The fin clip. A, Clipping the fin. B, Microscopic features in normal, black-pigmented fin, including fin rays (*R*) and epithelium (*E*), which covers the entire fin but is most easily seen on the edge of the fin. Numerous melanocytes (*M*) are also present. The cut edge of the fin is on the right in the *original* photo.

SKIN BIOPSY PROCEDURES: FIN CLIP

To fin clip (Fig. I-7, *A* and *B*), simply snip a small piece of one of the fins (the tail fin is usually the easiest) and prepare it as described for the skin scraping. This procedure is less traumatic than skin scraping because a cleaner and much smaller wound is produced; however, it is usually not as useful as a skin scrape. It may be difficult to see small pathogens such as *Ichthyobodo*, since the thick, hard fin rays prevent the preparation of a thin smear. The thinner parts of the smear should be searched.

EVALUATION OF SKIN BIOPSIES

Like all organ systems, the skin has a characteristic repertoire of reactions to injury. These can include hemorrhage, erosion, and ulceration (Fig. I-8, *A* and *B*). Fin ulceration (often termed ***fin erosion*** or ***fin rot***) is actually a gangrenous loss of tissue. It usually presents as a progressive necrosis starting at the tip of the fin. The leading edge of the lesion is often hyperemic or hemorrhagic. The necrotic tissue loses its normal color and often becomes white. Fragments of the fin rays may remain after the epithelium has sloughed, leaving a ragged appearance to the fin. Proliferation of epithelium may also occur concurrently with the progressive necrosis.

Another common response of the skin is hyperactivity of epithelium and goblet cells, which results in a thickening of the epithelium or increased mucus production that can give a cloudy appearance to the skin (Fig. I-8, *C*). Also, because the epidermis is not vascularized in small fish, there can be extensive epidermal damage without any bleeding. This may appear as depigmentation (Fig. I-8, *D*).

Numerous ectoparasites, bacteria, and other agents can incite these responses and often act together to produce lesions. Thus, the diagnosis of skin lesions can be complicated by the presence of several agents. Most ectoparasites can be present in low numbers on fish without causing

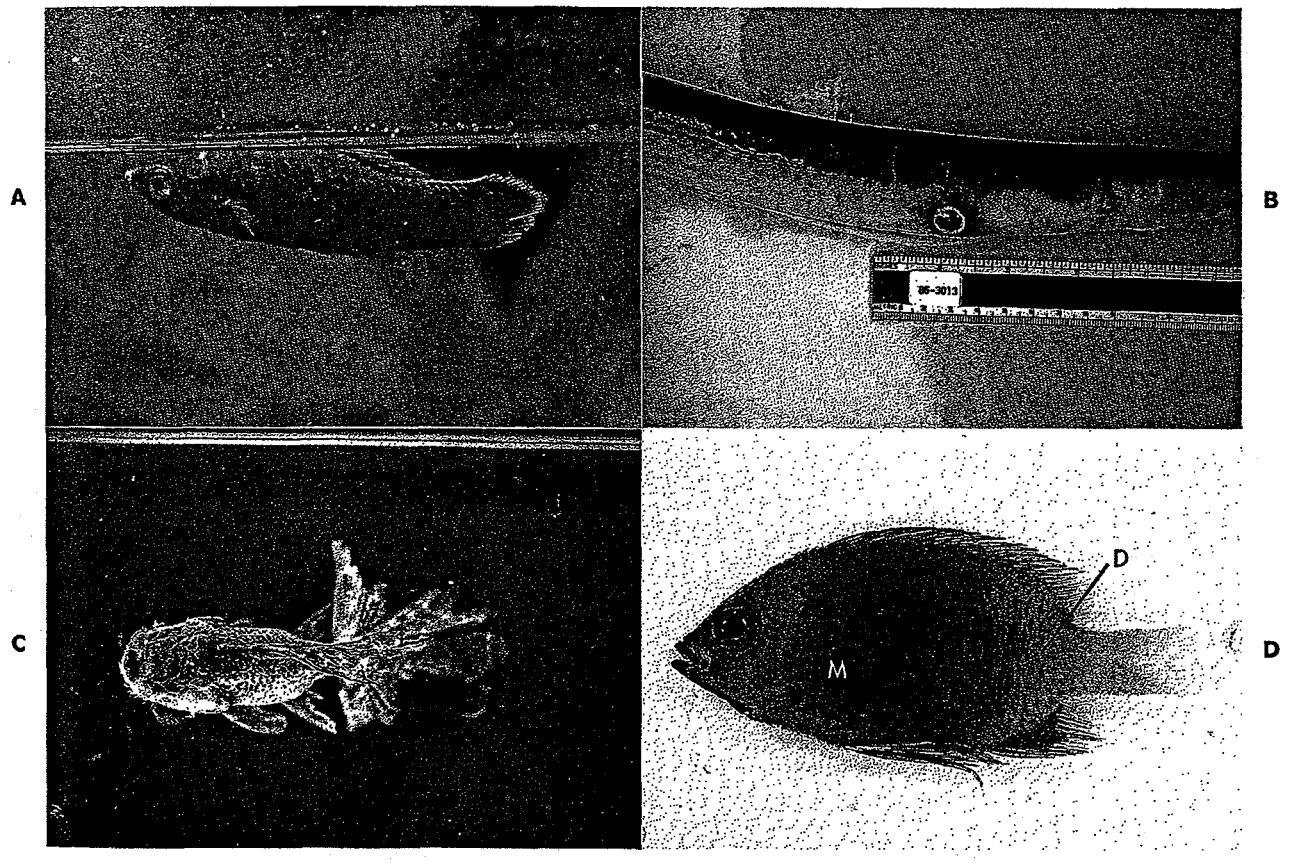

Fig. I-8 Common responses of the skin to damage. All of these responses are nonspecific and are thus only suggestive of certain problems. **A,** Caudal fin erosion and ulceration (*fin rot*). **B,** Skin ulcer. Note the hemorrhage around the ulcer. **C,** Cloudy appearance of the skin, with white flecks of detaching tissue; this may be due to epithelial hyperplasia and/or increased mucus production. **D,** Depigmentation (*D*) and melanization (*M*).

disease. For example, normal and apparently healthy channel catfish frequently have one or two trichodinids per low power field (MacMillan, 1985). Even such virulent pathogens as *Ichthyophthirius* (see *PROBLEM 19*) and *Amyloodinium* (see *PROBLEM 26*) can be carried asymptomatically. Conversely, heavy ectoparasitemia may be associated with systemic bacterial infections or other debilitating conditions. Thus, their significance depends on their concentration relative to other clinical findings.

It is important to determine the agent responsible for *initiating* a skin lesion to provide proper treatment. For example, water molds can colonize open skin wounds, and chronic ulcers often have many bacteria, especially motile rods, regardless of their primary etiology. However, even opportunists can kill fish, so treatment of secondary infections also is often advisable.

Many systemic diseases can have dermatological manifestations, although the etiological agents will often not be detectable in these dermatological lesions. Reddening of the fins and body (caused by congestion or hemorrhage) can be caused by gram negative bacteremias/septicemias, virus infections, or stressful environmental conditions (Smith & Ramos, 1976). Fish with mycobacteriosis (see *PROBLEM 53*) often have fin ulceration and faded coloration (Fig. I-8, *D*) (Reichenbache-Klinke, 1973).

Traumatic damage, such as that caused by aggressive tankmates (see *PROBLEM 87*), may mimic an infectious fin ulceration. Trauma is more likely to affect the more submissive members of a tank. Infectious agents are not present in purely traumatic lesions, although these may become secondarily infected.

Abnormal pigmentation may arise because of metacercarial infections (see *PROBLEM 55*) or other inflammatory lesions (e.g., *Ichthyophonus* (see *PROBLEM 67*) or *Mycobacterium* (see *PROBLEM 53*), or it may be a healing response to injury (Fig. I-8, *D*). Chronic inflammatory lesions often have large numbers of melanin-containing cells, including normal pigment cells (melanocytes) and inflammatory cells (melanomacrophages). These lesions should not be mistaken for melanotic cancers, which are much less common in fish.

GILL BIOPSY

Gill biopsy is a useful diagnostic tool in fish medicine. Many infectious agents that affect the skin can also infect the gills. Like the skin, the gill is a multifunctional organ; it is the major respiratory organ, is the primary site of nitrogenous waste excretion, and plays an important role in ionic balance. The complexity of the gill is reflected in its anatomical structure. Each gill arch has rows of macroscopically visible finger-like processes—the primary lamellae (Fig. I-9, *A* through *D*). Each primary lamella has rows of microscopic secondary lamellae. A capillary-like network of vessels in the secondary lamellae moves blood countercurrent to the water flow, facilitating gas exchange.

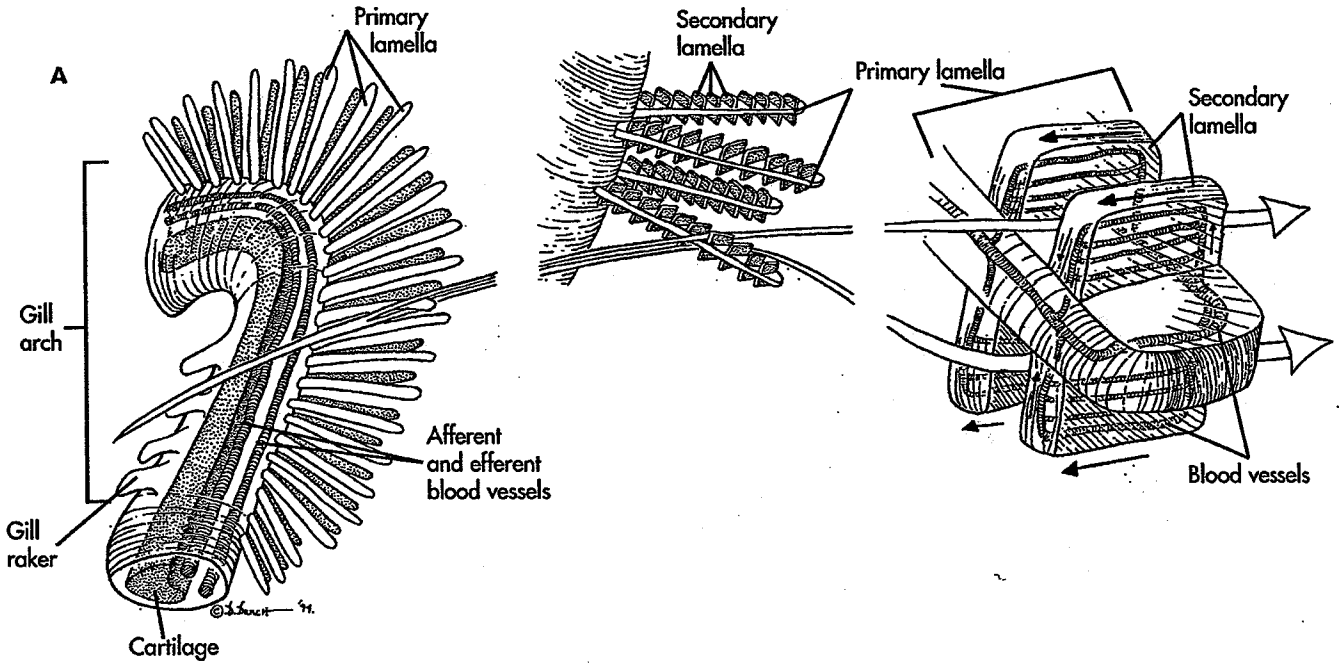

Fig. I-9 A, Diagram of normal gill. *Light arrows* indicate direction of water flow; *dark arrows* indicate blood flow.

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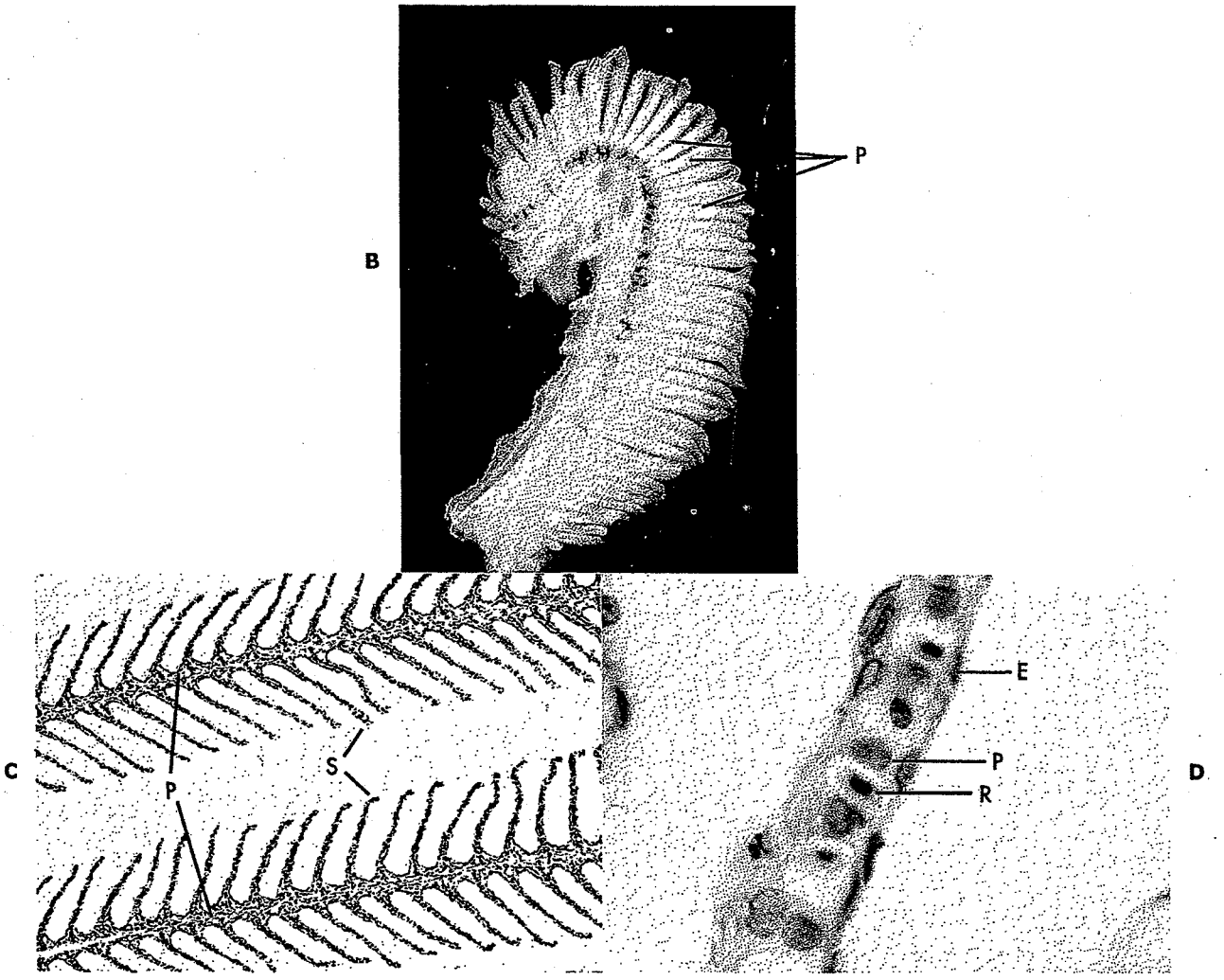

Fig. I-9—cont'd. **B,** Low-power microscopic view of a formalin-fixed gill arch, showing primary lamellae (*P*), each having rows of secondary lamellae. (Compare with Fig. I-10, *C*.) **C,** Low-power histological section of normal gill. *P* = primary lamella; *S* = secondary lamella; hematoxylin and eosin. **D,** High-power histological section of normal gill secondary lamella. *R* = red blood cell; *P* = pillar cell; *E* = epithelial cell; hematoxylin and eosin.

GILL BIOPSY PROCEDURE

Immediately before biopsy the gills should be examined grossly. Healthy gills are bright red. Pale pink gills suggest anemia, while pale tan gills suggest methemoglobin formation (see *PROBLEM 5*). Do not confuse anemia with postmortem change (gills quickly become pale pink after death because of passive drainage of blood from the gills). Because the thymus is grossly visible in the gill chamber, it can also be evaluated at the same time. It should be glistening white (see Fig. I-32). Thymic hemorrhage has been associated with stress in salmonids (Goede & Barton, 1990).

Gill biopsy (Fig. I-10, *A* through *H;* Fig. I-11, *A* and *B)* is performed by inserting the tip of a pair of fine (e.g., iridectomy) scissors into the gill chamber. The scissors are then gently opened, lifting the operculum until the gill arches can be seen. The tips of several primary lamellae are then cut and transferred to a slide; a coverslip is then applied. Only the tips of the lamellae should be cut; bleeding should be minimal.

EVALUATION OF GILL BIOPSIES

The most common response of the gill to damage is hyperplasia and hypertrophy of epithelial cells, which can eventually lead to fusion of adjacent secondary or even primary lamellae. This severely reduces gas exchange at the wound site and can lead to respiratory distress. This can occur because of injury from bacteria or parasites or

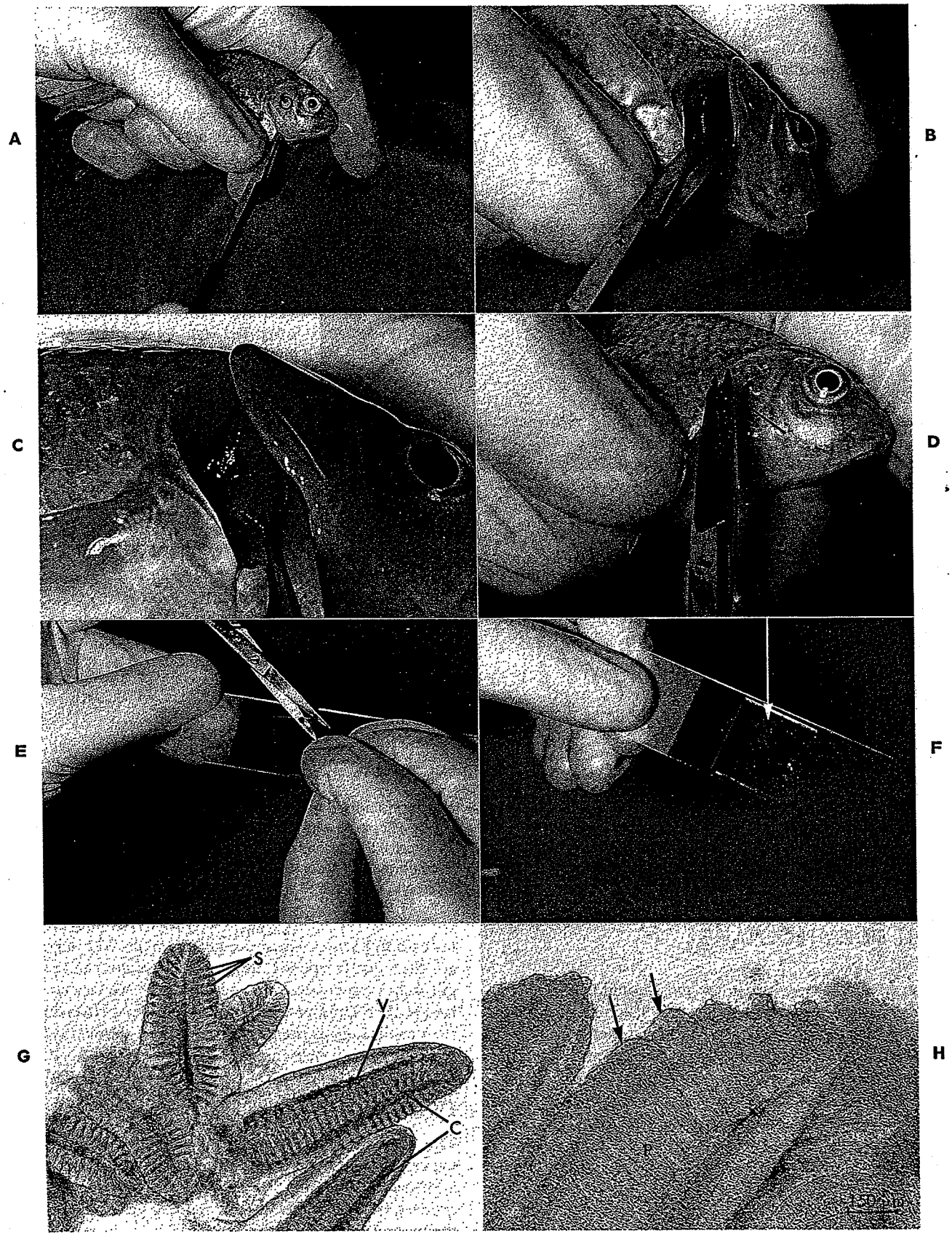

Fig. 1-10 *For legend see opposite page.*

Fig. I-10 The gill biopsy. **A,** Using a fine pair of scissors to pry open the gill cover, or operculum (*O*). **B,** Inserting the scissors under the tips of the gills just before cutting the tips. **C,** Close-up of Fig. I-10, *B*. Each horizontal, finger-like strip of tissue is a primary lamella. Only the distal tips of the primary lamella should be cut. **D,** Gill tissue on the scissor (*arrow*) after being excised from the gill. **E,** Scraping the biopsy material onto the slide with a coverslip. **F,** Gill tissue (light material at the *arrow*) covered with the coverslip. **G,** Low-power photomicrograph of biopsy of a normal gill. The large finger-like structures are primary lamellae. *S* = secondary lamellae; *C* = cartilage support of primary lamella; *V* = blood vessels. **H,** High-power view of normal gill, showing secondary lamellae (*arrows*). Individual secondary lamellae may not be visible in some squashes of normal gill, depending on how the tissue lies. *P* = primary lamella.

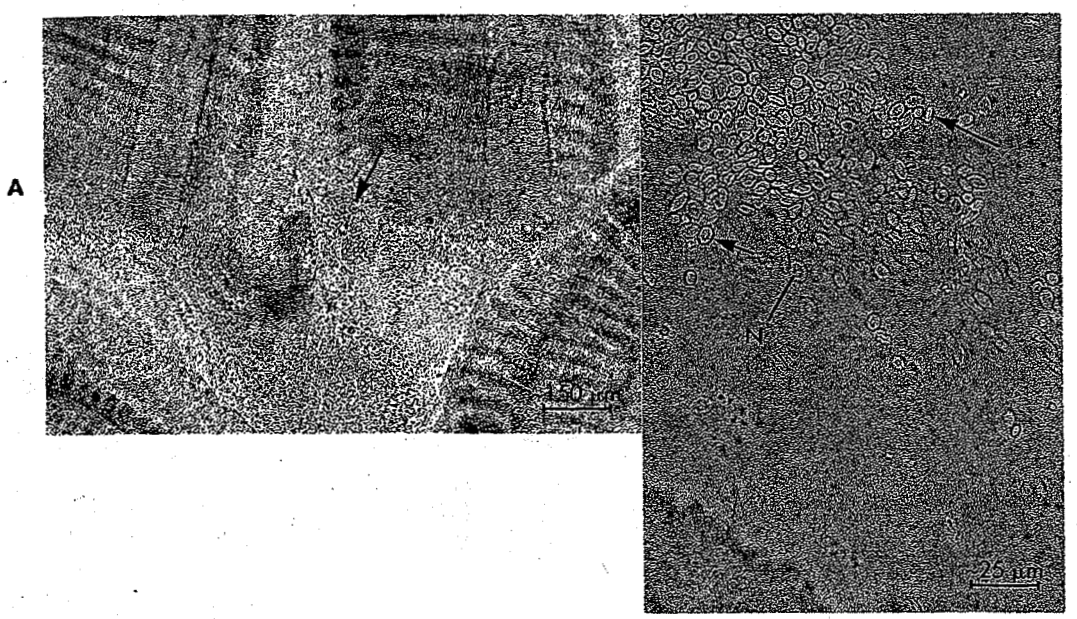

Fig. I-11 Blood cells in a wet mount of gill. **A,** The cells (*arrow*) are streaming from the cut surface of the gill. **B,** High-power view showing individual red blood cells. Key characteristics include oval shape in top view (*small arrow*) and laterally compressed in side view (*large arrow*). Nucleus (*N*) gives cells a fried-egg appearance.

(*A* and *B* photographs by L. Khoo and E. Noga.)

from poor water quality. Hyperplasia and hypertrophy can result from the feeding activity of protozoa such as *Trichodina* (see *PROBLEM 21*), *Chilodonella* (see *PROBLEM 22*), or *Ichthyobodo* (see *PROBLEM 28*) (Wooten, 1989). Some parasites, such as *Ichthyophthirius* (see *PROBLEM 19*) and *Amyloodinium* (see *PROBLEM 26*), induce focal hyperplasia at their attachment sites (see Fig. II-19, *D*). Some bacterial pathogens produce substances that stimulate epithelial proliferation. Epithelial hyperplasia and lamellar fusion have also been documented in vitamin deficiencies (see *PROBLEM 79*). Changes in gill structure are most easily recognized in histological sections, but if gill hyperplasia is detected on a wet mount it indicates that serious damage is present (Fig. I-12, *A* and *B*).

As on the skin, many pathogens may be present in low numbers on the gill without causing clinical disease; thus, interpretation of their significance depends upon other clinical findings.

A common sequela of gill infections is telangiectasis, or the dilatation of groups of small blood vessels on the secondary lamellae (Fig. I-12, *C*). This condition can also result from a number of environmental toxins. Telangiectasis can also be iatrogenically induced in some fish by cranial concussion (Herman & Meade, 1985). Frank necrosis of gill tissue (*gill rot*) is characterized by the destruction of secondary lamellae and, in severe cases, the stripping of gill tissue down to the cartilaginous skeleton of the primary lamellae. It can be caused by pigmented bacteria and various toxins.

Because the gill is highly vascularized, lamellar biopsy can also be used to examine the blood in fish that are too small to be bled by conventional means (Fig. I-11), allowing the detection of hemoparasites or other pathogens.

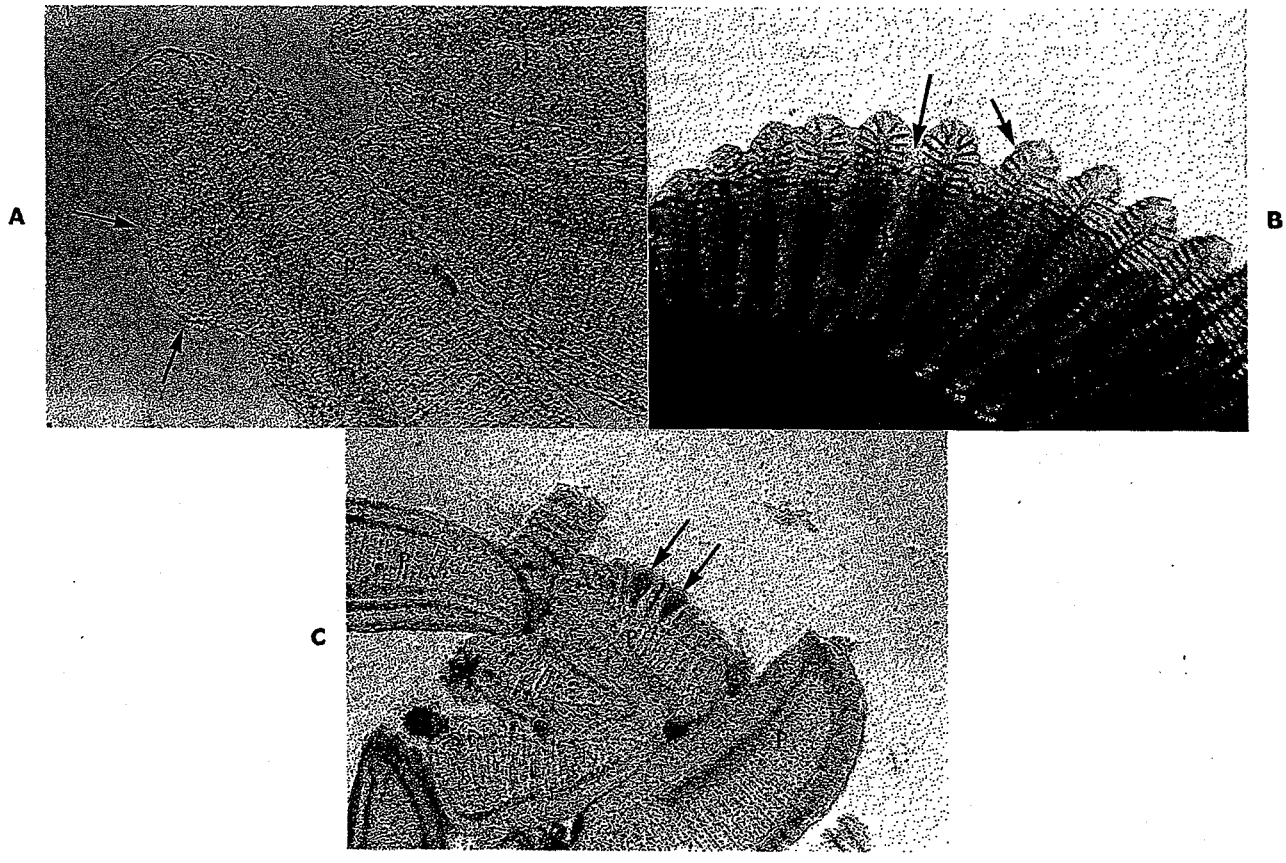

Fig. I-12 Examples of gill lesions visible in wet mounts. **A,** Focal epithelial hyperplasia (*arrows*), protruding above the surface of the primary lamella (*P*). **B,** Severe epithelial hyperplasia, with resultant fusion of lamellae to one another. There is both fusion of adjacent secondary lamellae (*small arrow*) and fusion of adjacent primary lamellae (*large arrow*). **C,** Telangiectases (*arrows*) or weakening of blood vessel wall of a secondary lamella, causing vessel dilation. *P* = primary lamella. (*B* photograph courtesy of A. Mitchell.)

FECAL EXAM

A fecal exam may identify helminth ova (especially nematodes and also digeneans) and some protozoans (e.g., *Hexamita*). Fecal material can be obtained by siphoning debris from the bottom of the tank. This is not stressful to the fish; however, it is least sensitive for diagnosis. Samples are also contaminated with many nonpathogenic organisms. A fecal sample can often be obtained by anesthetizing a fish. Standard sodium nitrate flotation can be used for concentrating samples from fecal matter or aquarium debris (Langdon, 1992a).

BLEEDING FISH

Hematology and clinical chemistry are not routinely used for fish disease diagnosis, although they can be useful in some circumstances. Anemia in fish is often easily detected by examining the gills, which are a pale pink color (rather than a normally bright red color) if anemia is present. Blood samples should always be taken if fish are anemic.

Fish that are less than 8 cm (3 inches) usually cannot be bled without risk of killing them, so this technique is not feasible for small fish that cannot be sacrificed.

Anticoagulants

If blood is to be obtained for simply determining hematocrit or for making routine blood smears to look for hemoparasites or bacteremia, standard mammalian blood collection procedures are satisfactory. Heparin is usually an effective anticoagulant when used at ~50 to 100 USP Units/mL. However, heparin, which inhibits thrombin, will not prevent coagulation if clotting has begun (i.e., if a small clot is present in the sample because of blood vessel damage during sampling), because coagulation can proceed via an alternate pathway; this is a common prob-

lem in fish because of their small vessels. Ethylenediamine tetraacetic acid (EDTA) at 4 to 5 mg/ml final concentration will totally prevent clotting by chelating required divalent cations. However, using EDTA in combination with tricaine sedation is not recommended because it inexplicably causes hemolysis in many cases. This hemolysis problem can be reduced by cooling the blood to 4° C and/or rapidly preparing smears and removing plasma from the cells.

Blood Separation and Analysis

If detailed cellular or chemical analyses are to be performed (e.g., differential counts, enzyme measurements, etc.), the clinician should optimize the conditions for the fish species that are being examined because various researchers have noted problems under a wide range of conditions that are routinely used in mammalian hematology. The most important variables are type and concentration of anticoagulant and type of anesthetic. It is best to avoid using chemical anesthesia. Stun fish if possible. Samples should be analyzed as quickly as possible. Significant changes often occur in whole blood after 1-hour storage at room temperature and can occur 1 to 3 hours after refrigeration (Houston, 1990).

Plasma and/or serum should be rapidly separated from cells and frozen at the lowest possible temperature. For serum, it is usually best to allow the sample to clot at room temperature for 5 minutes, refrigerate it for 1 to 2 hours, rim the clot, and then centrifuge to separate the serum from cells. Pediatric serum separator tubes (Becton-Dickinson) are useful because they handle small volumes.

All fish blood cells, including erythrocytes and thrombocytes (platelet analogue) are nucleated, which prevents the use of automated white cell counting or differentiation. Total white cell counts must be done by staining the white cells and then counting them with a hemacytometer (see Box I-1). Differential counts are obtained from blood smears.

Preparing Blood Smears

Blood smears are prepared as are routinely done for mammals. Smears should be made quickly; drying smears with a hair dryer is desirable. Commercial differential stains (e.g., Diff-Quik, Baxter Diagnostics, Inc.) are suitable for most diagnostic purposes (Fig. I-13). White blood cell morphology varies greatly between different fish species.

Bleeding with Needle and Syringe

For larger fish, a needle and syringe with anticoagulant can be used to bleed the fish from one of several sites. One of the least traumatic sites for collecting blood is the caudal vessels. This site can be approached laterally or ventrally. After the fish is sedated, the needle is gently pushed through the skin near the base of the caudal peduncle. After contact is made with the vertebral column, which is felt as firm resistance, the needle is directed slightly ventrally and lateral to the vertebral

BOX I-1

METHOD FOR STAINING BLOOD FOR WHITE BLOOD CELL COUNTING*

Step 1. Prepare Natt-Herrick's stain as follows:

Sodium chloride ($NaCl$)	3.88 g
Sodium sulfate ($NaSO_4$)	2.50 g
Sodium phosphate (Na_2HPO_4)	1.74 g
Potassium phosphate (KH_2PO_4)	0.25 g
Formalin (37 percent)	7.50 ml
Methyl violet	0.10 g

Bring to 1000 ml with distilled water and filter through Whatman #10 medium filter paper.

Step 2. Prepare a 1:200 dilution of blood with Natt-Herrick's stain (add 20 µl blood to 4 ml of Natt-Herrick's stain).

Step 3. Mix well, and leave at room temperature for 5 minutes; then fill both sides of a hemocytometer with the stained blood.

Step 4. After 5 more minutes, perform a white blood cell count, using the 10X objective. That is, count all white blood cells in the 4 large corner squares on both sides of the hemocytometer chamber (the counts within each square should be within 10 percent of each other). Add all 8 counts together and use this total count to calculate:

$$\frac{\text{Total \# WBCs counted}}{8} \times 2000 = \text{\#WBCs/µl of blood.}$$

All white blood cells (leucocytes + thrombocytes) will stain dark violet, distinguishing them from red blood cells. It is usually not possible to easily distinguish thrombocytes from leucocytes.

*Modified from procedure of T. Laws. Personal Communication.

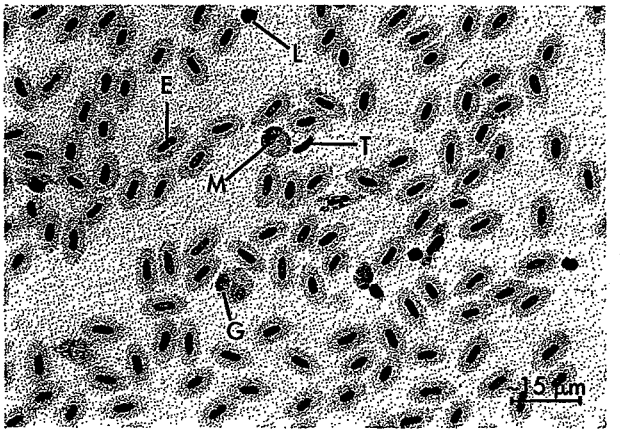

Fig. I-13 Blood smear from a normal goldfish. Blood cell morphology varies greatly among different species. If used, smears must be compared with those from known, healthy individuals. Erythrocyte (*E*); thrombocyte (*T*); lymphocyte (*L*); monocyte (*M*); granulocyte (*G*). Modified Wright's stain.
(Photograph by L. Khoo and E. Noga.)

column, while the syringe gently aspirates (Fig. I-14, *A* and *B*). It may be necessary to slowly rotate the needle before blood can be withdrawn. When one of the caudal vessels is entered (either artery or vein; they run closely together), blood is aspirated. Filling the hub of the needle is a sufficient amount for making a blood smear.

Larger fish may also be bled from the heart. The heart is usually located near the posterior edge of the gill chambers (Fig. I-15). The heart may also be approached dorsally by directing the needle into the posterior portion of the gill chamber. Bleeding the heart is probably more traumatic and potentially more dangerous than bleeding the caudal vessels. Less commonly used anatomical approaches are discussed by Houston (1990).

Bleeding by Capillary Tube

This method is used to bleed small fish (less than 8 cm or 3 inches). The fish is anesthetized and then placed on a smooth, flat surface. The base of the tail is then severed with a scalpel blade (Fig. I-16). A heparinized capillary tube is quickly applied to the caudal vessels, and the blood is collected in the tube by capillary action. A blood smear is then made immediately and is stained, using standard methods. This method probably results in significant tissue fluid contamination, which should be considered if samples are used for clinical chemistry. The fish should be euthanized immediately.

BIOPSY OF INTERNAL ORGANS

The kidney is the preferred site for isolation of many viral and bacterial diseases in fish (Amos, 1985;

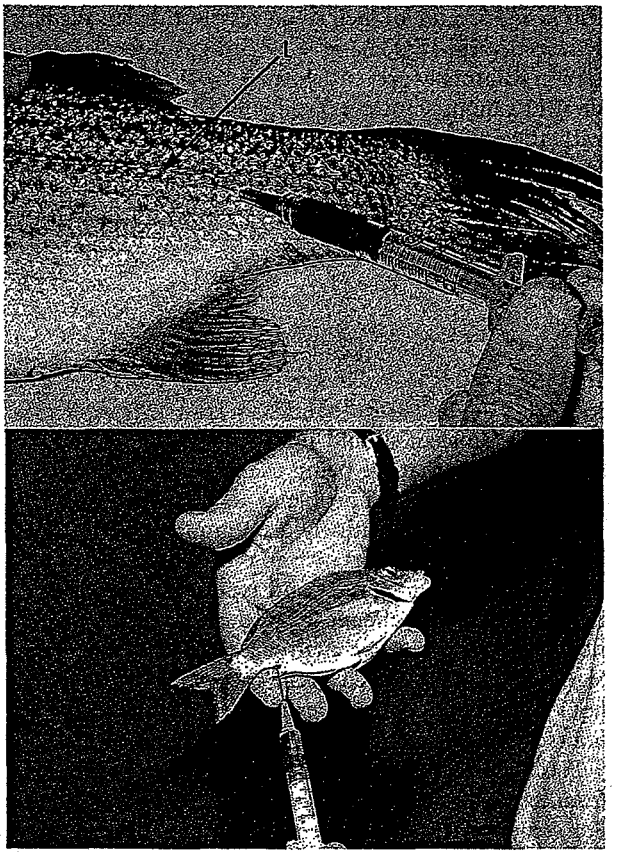

Fig. I-14 Bleeding from the caudal vein by needle and syringe. A, Location for inserting the needle, using the lateral approach. *L* = lateral line. B, Location for inserting the needle, using the ventral approach.

Thoesen, 1994). However, if valuable fish are involved (e.g., broodstock), it may not be desirable to sacrifice such fish to determine their health status. An alternative, nonlethal method involves biopsy (Noga et al., 1988, *B*). In teleost fish, the kidney is a long, ribbon-shaped organ that runs retroperitoneally along the length of the peritoneal cavity. Because it is composed of hematopoietic, as well as excretory tissues, the kidney does not have the solid structure of normal mammalian renal parenchyma; instead it has the consistency of bone marrow. At its cranial limit, the kidney curves ventrally and lies just beneath the medial surface of the branchial chamber. This makes it accessible to needle aspiration.

The fish to be biopsied either is anesthetized or is restrained by another person. The gill operculum is lifted, and a 3 cc syringe with a 22 gauge, 1.5 inch needle is directed dorsally and then dorsocaudally into the kidney, just caudal to the last branchial arch (Fig. I-17, *A*). The syringe is then aspirated until 0.10 ml of kidney tissue is collected, filling the hub of the syringe. In

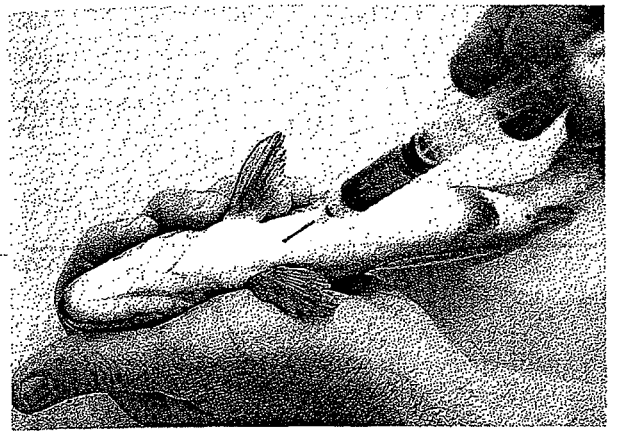

Fig. I-15 Bleeding a rainbow trout from the heart.

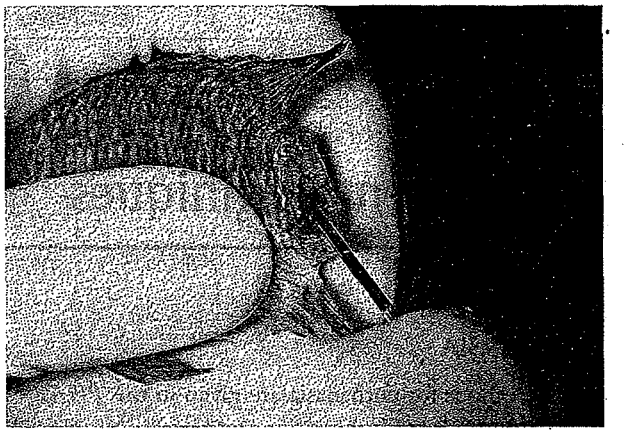

Fig. I-16 Bleeding from the caudal vein by severing the tail. After anesthetization, a sharp scalpel is used to cut off the base of the tail. A heparinized capillary tube is immediately applied to the vessel until sufficient blood is obtained.

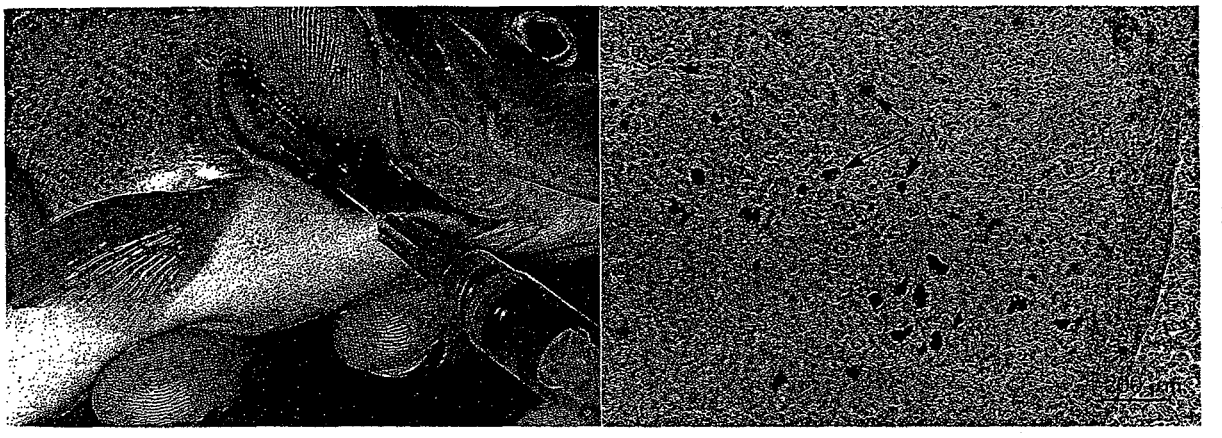

Fig. I-17 Anterior kidney biopsy technique. A, Inserting a needle through the medial membrane of the gill chamber and into the kidney. O = operculum. B, Confirmation that kidney material has been obtained as indicated by the presence of melanocytes (*M*) in a wet mount of biopsy material.

(From Noga et al., 1988.)

salmonids, the presence of kidney tissue can be rapidly confirmed by examining a small portion of the aspirate microscopically and confirming that tissue fragments and melanocytes are present (Fig. I-17, *B*).

This technique is as effective as standard necropsy culture in diagnosing enteric redmouth disease in rainbow trout (Noga et al., 1988b) and would probably be useful for diagnosing other infectious diseases in salmonids. Its usefulness in other fish species remains to be determined and is probably limited to fairly large fish (probably those that are at least 15 cm or 6 inches long). Note that not all fish have melanocytes in the anterior kidney, and thus only tissue fragments may be seen. Other techniques that involve minor surgical procedures to nonlethally sample posterior kidney and liver have also been developed. See Wooster et al. (1993) for details.

CHAPTER 5

Postmortem Techniques

EUTHANASIA

Proper methods for euthanasia are given in the *PHARMACOPOEIA.*

PRESERVING PARASITES

Live specimens are always preferable for diagnosis, but if assistance is needed for identification and live material cannot be sent to a reference laboratory, samples need to be properly preserved. Table I-4 describes procedures for properly preparing specimens.

Many protozoa can be identified in histological sections, but many can detach from skin or gills with fixation and processing. It is best to fix the gills together, rather than cutting them into individual filaments, especially when loosely attached parasites (e.g., *Chilodonella*, *Trichodina*) may be present. Protozoa can be smeared on a slide, air dried, and stained—the same as for blood smears (see Fig. II-20, *E*), but this technique is rarely used for identifying protozoa in clinical material. Techniques for preserving metazoan parasites are more commonly used.

CULTURING FOR BACTERIA

It is often desirable to refer fish to a regional reference laboratory if bacterial disease is suspected because the techniques required to properly identify bacterial pathogens of fish are somewhat specialized. Samples should be submitted to a laboratory that is familiar with culturing bacteria from aquatic species because many aquatic pathogens have special requirements. For example, it is best to culture fish isolates at room temperature (22° to 25° C), not 37° C, as is routinely done in commercial microbiology labs, since some fish pathogens grow poorly or not at all at 37° C. For *Vibrio salmonicida*, samples should be incubated at 17° C. Samples from marine fish should be cultured on a medium that has a high salt content (e.g., trypticase soy agar with 2% NaCl) or on a nutrient-rich blood agar, such as Columbia agar with 5% defibrinated sheep blood (CBA). CBA is a good general-purpose medium for both freshwater and marine bacterial pathogens.

Some bacteria require other, specialized media, but these media are not routinely used in the clinical work-up. Selective media can also be used to enhance the isolation of certain pathogens but would not be routinely used in a clinical work-up unless prior knowledge of pathogens likely to be encountered warranted it. Not all differential media used for freshwater organisms may be reliable in estuarine environments. For example, Rimmler-Shotts (Shotts & Rimmler, 1973), a useful, selective medium for identifying *Aeromonas hydrophila* in freshwater, cannot differentiate between *A. hydrophila* and non-01 vibrios in estuarine waters (Kaper et al., 1981). See Shotts and Teska (1989) for various selective media used for bacterial isolation.

Samples may be submitted to a laboratory in one of several ways (Table I-5). Live specimens should be used for culture whenever possible. The only exception is when the only fish displaying clinical signs are dead (i.e., all of the live fish appear healthy). Identification of an obligate pathogen (e.g., *Aeromonas salmonicida*) in a dead fish is a stronger diagnosis than the isolation of an opportunist (e.g., *Aeromonas hydrophila*), especially if large numbers are present.

Whole fish may be frozen and shipped to the laboratory on dry ice. The recoverability of many common bacterial fish pathogens ranges from 20 to 60 days when samples are frozen at −20° C, which is the temperature of a home freezer (Brady & Vinitnantharat, 1990). Immunodiagnosis is increasingly being used for rapid, presumptive identification of pathogens (e.g., bacterial kidney disease [BKD]; see *PROBLEM 52*). While spleen, liver, and peritoneal fluids are common culture sites, the organ of choice for isolating systemic bacterial pathogens in fish is the kidney, which can be approached dorsally or ventrally (Figs. I-18, *A* through *H*, and I-19, *A* through *D*).

Dorsal Approach to Kidney

The fish is euthanized and the dorsal fin is clipped off. The surface of the back is decontaminated either by swabbing the area with antiseptic (e.g., quaternary ammonium or 70% alcohol) or by searing the skin with a

Table I-4 Recommended methods of preserving parasites for future identification.

Parasite group	Relaxation procedure	"Relaxed" parasite	Fixation	Storage	Final preparation for identification
Monogeneans/ digeneans*	None usually needed for small worms; gently flatten under a coverslip, and flood slide with fixative for 5 minutes	Not contracted; allows some expulsion of eggs from uterus	Hot (55° to 65° C) AFA or hot NBF	AFA or ETOH	Stained and permanently mounted in mounting medium
Cestodes*	Cold (4° to 8° C) water or saline for 1 to 12 hours; gently flatten under a coverslip, and flood slide with fixative for 5 minutes	Not contracted; allows some expulsion of eggs from uterus	Hot AFA or hot NBF or hot ETOH	AFA or ETOH	Stained and permanently mounted in mounting medium
Nematodes*	None usually needed for small worms; stretch large worms by holding each end of the worm with forceps, and add fixative for 5 minutes	Completely uncoiled	Hot AFA or hot ETOH	AFA or ETOH or glycerol: ETOH	Small nematodes can be cleared in glycerol: ETOH and mounted permanently in glycerol jelly; large nematodes are cleared and temporarily mounted in glycerol: ETOH
Acanthocephalans	Cold (4° to 8° C) water or saline for 1 to 12 hours	Proboscis fully extruded	Hot AFA or hot HBF or hot ETOH (puncture cuticle)	AFA or ETOH	Small: stained and mounted; large: unstained and mounted in glycerol: ETOH
Hirudineans	Tricaine; sodium pentobarbitol	Not contracted	Hot ETOH	ETOH	Small: stained and mounted; large: glycerol: ETOH
Arthropods	Not required	Not required	Cold (4° to 8°C) ETOH	ETOH	Unstained and cleared in 10% KOH or Hoyer mounting medium
Ciliates†	N/A	N/A	Air-dry smear‡	Stain immediately	Stained with Wright's or Klein's silver stain (Lom and Dykova, 1993) and permanently coverslipped with mounting medium (Permount or equivalent)
Flagellates†	N/A	N/A	Air-dry smear‡	Stain immediately	Stained with Wright's, and permanently coverslipped with mounting medium (Permount or equivalent)
Amoebae†	N/A	N/A	Air-dry smear‡	Stain immediately	Stained with Wright's, and permanently coverslipped with mounting medium (Permount or equivalent)
Myxozoa†	N/A	N/A	Air-dry smear‡	Stain immediately	Stained with Wright's, and permanently coverslipped with mounting medium (Permount or equivalent)
Microsporidians†	N/A	N/A	Air-dry smear‡	Stain immediately	Stained with Wright's, or Gram's, and permanently coverslipped with mounting medium (Permount or equivalent)

Modified from Smith & Noga, 1993.
AFA=alcohol-formalin-acetic acid; NBF=10% neutral buffered formalin; ETOH=70% ethanol; glycerol: ETOH=100% glycerol: 70% ethanol.
*Before beginning preservation procedures, encapsulated larvae should be manually dissected out of the capsule or the capsule should be digested with 0.2% pepsin in 0.1 M HCl.
†See problem list for other techniques used in diagnosis.
‡Note that this procedure is less reliable for protozoan identifcation than routine histopathology but can be useful when submitting specimens to reference laboratories for identification.

Table I-5 Diagnostic usefulness of different tissue preservation techniques for identifying fish pathogens.*

Specimen	Protozoan ectoparasites†	Monogenean ectoparasites†	Metazoan parasites (except Monogenea)†	Myxozoa and Microsporea†	Viral isolation	Bacterial isolation	Histologic value
Live fish	+++	+++	+++	+++	+++	+++	+++
Dead fish‡	−	−	++	++	+	−	−
Iced fish§	+	++	+++	+++	++	+	+
Frozen fish‖	+	+	++	++	++	++	+
Fixed fish¶	++	+	+	++	−	−	+++

*The ability to recover various pathogens varies greatly; these comparisons are only intended as general guidelines. +++ = best; − = virtually useless.
†Comparisons between live, dead, iced, and frozen fish are based upon the ability to identify parasites in wet mounts; diagnostic usefulness of fixed fish is based upon the ability to identify pathogens in histological sections.
‡Dead fish left in water at room temperature for 6 to 12 hours.
§Live fish placed in a plastic bag on wet ice for 6 to 12 hours.
‖Live fish placed in a plastic bag frozen at −20° C.
¶Tissues from live fish immediately placed in 10% neutral buffered formalin.

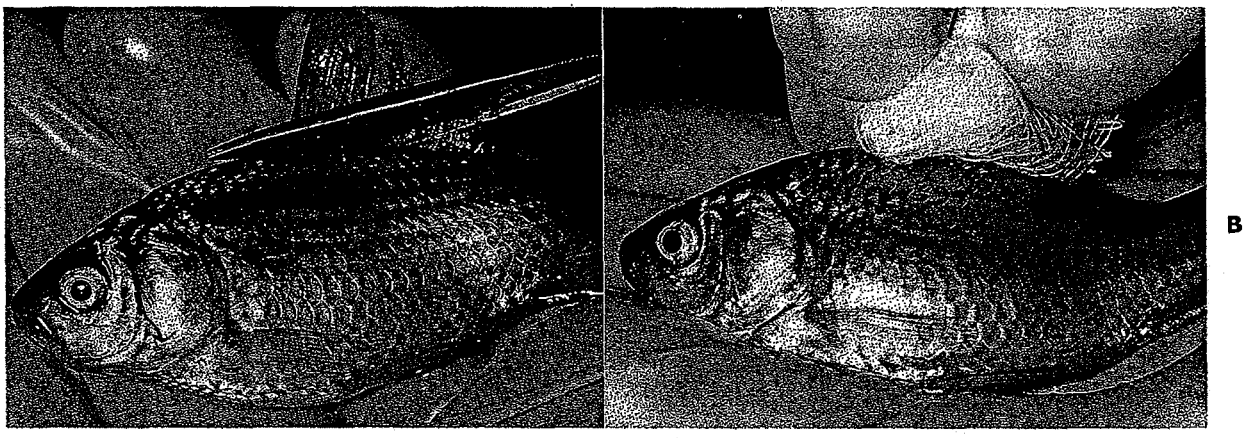

Fig. I-18 Culturing for bacteria, using the dorsal approach. A, After anesthetization the dorsal fin is clipped to reduce possible contamination. B, The surface of the back is decontaminated with antiseptic and then dried with a dry, sterile gauze pad.

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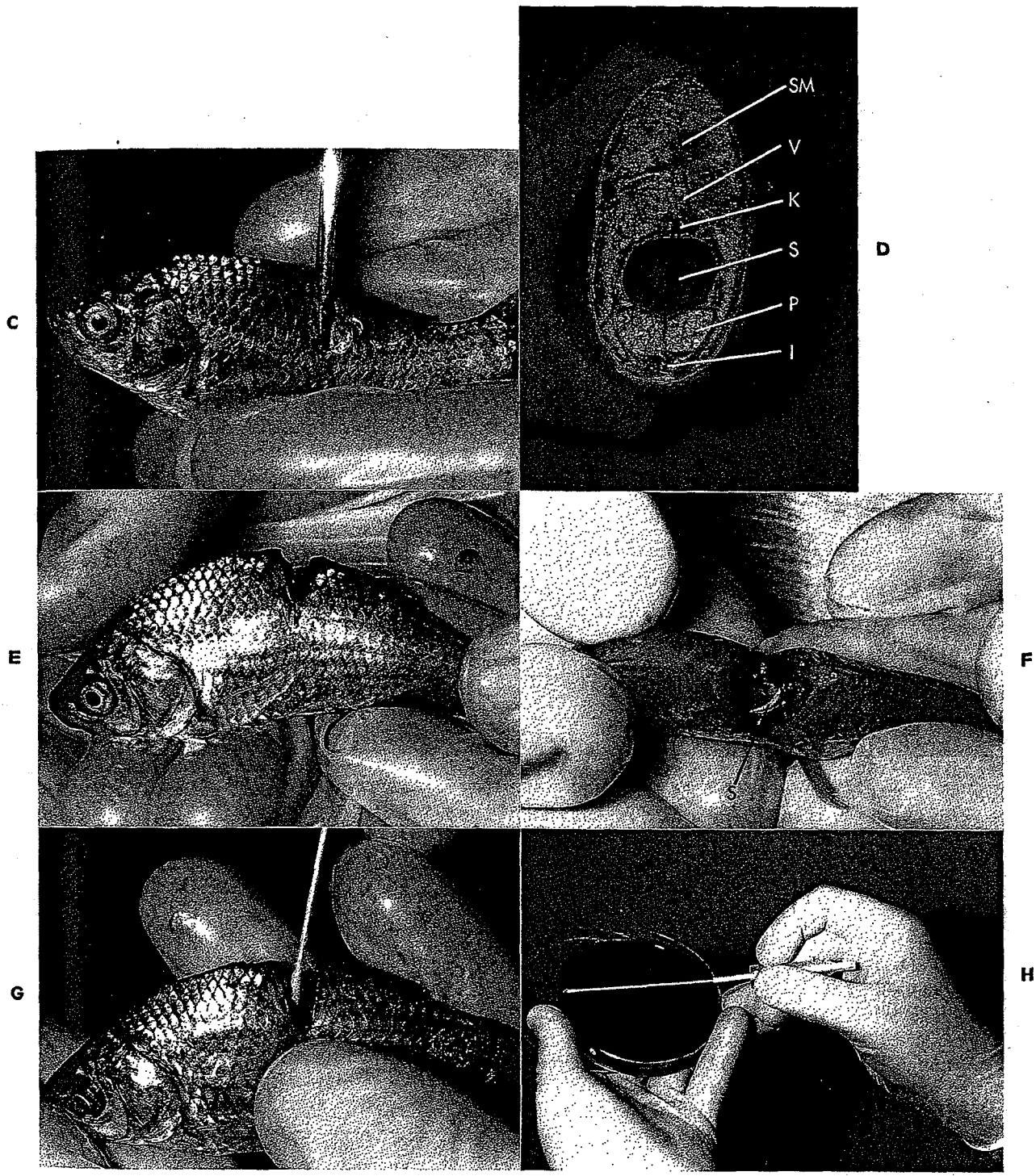

Fig. I-18—cont'd. C, The back is cut with sterile scissors. Care is taken not to cut so far as to enter the peritoneal cavity. This step is the most likely time for contamination to occur. D, Whole-body cross-section through a fish. Note that the kidney (*K*) is ventral to the vertebral column (*V*), which must be severed before reaching the kidney. The swim bladder (*S*) is ventral to the kidney. *P* = viscera in the peritoneal cavity, including intestine (*I*). *SM* = skeletal muscle. E, Reflecting the body ventrally (fish in Fig. I-18, *C*) to expose the kidney for culture. F, Entrance into the kidney is indicated by the appearance of a large amount of hemorrhage because of the highly vascular nature of the kidney. The collapsed, white swim bladder (*S*) lies ventral to the kidney; it is not clearly visible on all fish. G, Touching a sterile Culturette to the kidney and being careful not to touch other areas, which would cause sample contamination. H, Inoculating a Columbia blood agar plate with the sample, using a Mini-tip Culturette (Becton-Dickinson) and spreading the inoculum.

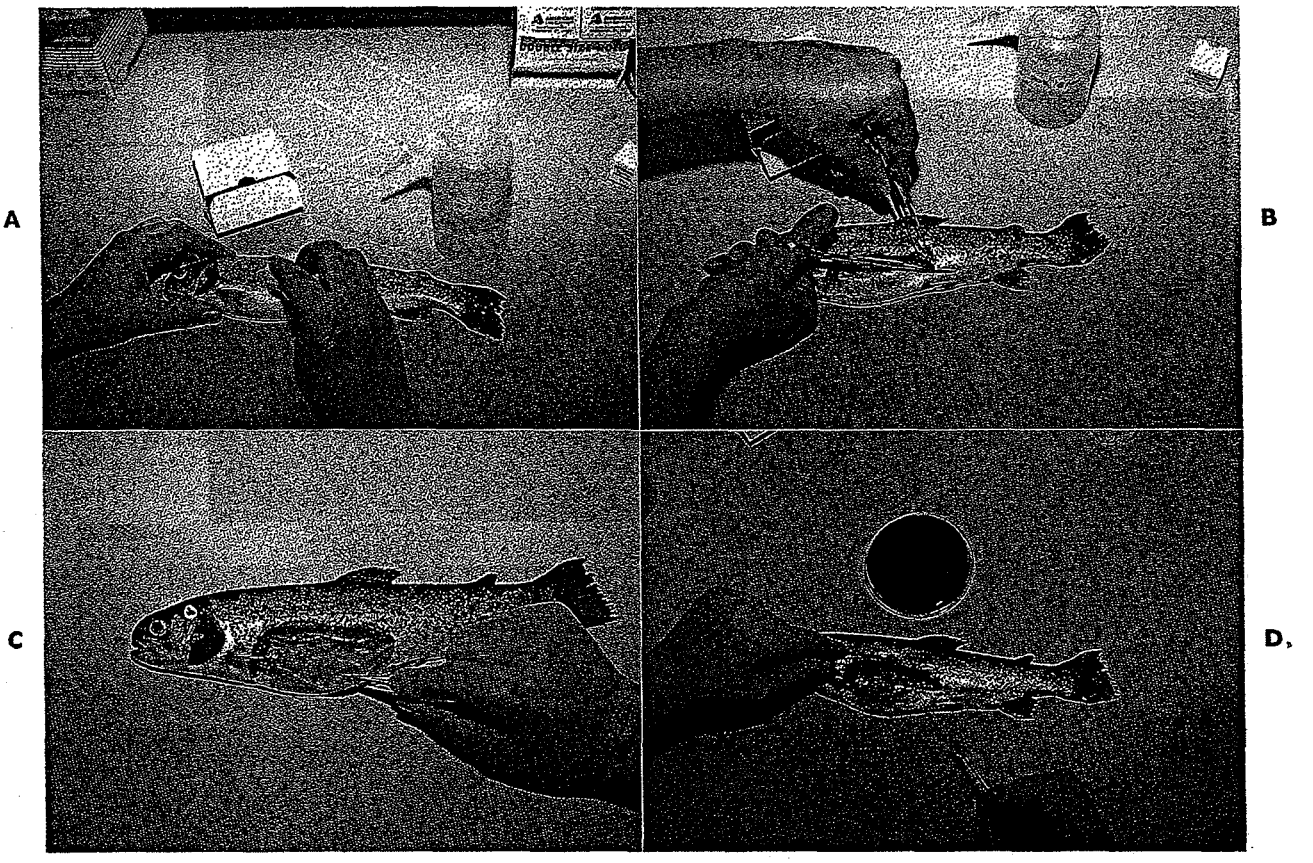

Fig. I-19 Culturing for bacteria, using the ventral approach. A, After anesthetization the flank is swabbed with antiseptic, avoiding the anus and any skin lesions. The area is then dried with a dry sterile gauze pad. B, The body wall is cut with sterile scissors. Care is taken to avoid the anus and to cut close to the body wall to prevent severing the intestine. C, Viscera are aseptically reflected, exposing the swim bladder (also see Fig. I-18, *E*). The swim bladder must be cut or reflected to reach the kidney. D, The kidney is often covered by a tough fibrous capsule, which must be severed to enter the parenchyma.

flat, metal object (e.g., knife or spatula) heated in a flame. If an antiseptic is used, the skin should be wiped dry with sterile gauze. Sterile scissors are then used to cut into the decontaminated area. The incision should be made just deep enough to cut through the vertebral column. Cutting deeper may enter the peritoneal cavity, possibly rupturing the intestines and contaminating the sample.

The exact incision site varies slightly, depending on the species, but generally, an incision is made just posterior to the dorsal fin. Another useful landmark is to cut at one quarter of the distance from the anus to the posterior edge of the operculum. Once the incision has been made, the head and tail of the fish should be bent downward (ventrally) to expose the kidney (see Fig. I-18, *E*). The kidney lies immediately beneath the vertebral column and appears as a dark red, bloody area (see Fig. I-18, *F*). If the incision is not deep enough, that is, if the incision is only into the epaxial (upper body) muscles, almost no blood will be present, since muscle has much less blood supply than the kidney. Once the kidney is exposed, a sample then can be taken with a sterile loop or a Culturette (Becton-Dickinson Microbiological Systems).

Ventral Approach to Kidney

The fish is swabbed with antiseptic, avoiding the anal area, and placed in lateral recumbency. The peritoneal cavity is opened, using aseptic technique, and the body wall is cut away (see Fig. I-19, *B*). The kidney is reached by gently pushing the viscera in the peritoneal cavity to one side and deflecting the swim bladder away from the vertebral column. This part of the procedure is best done with a sterile, blunt probe. The kidney runs the entire length of the peritoneal cavity, just ventral to the vertebral column. Note that on large fish, you may need to use a scalpel to cut the membrane that separates the kidney from the swim bladder. A loop or swab may be used for culture. This material can then be immediately

streaked onto a culture plate or shipped on ice to a diagnostic laboratory if it is placed in a transport medium. Alternatively, a piece of kidney may be removed and placed in a sterile syringe barrel or red-top Vacutainer (Becton-Dickinson) tube; unless it is plated immediately, the specimen should be frozen for shipment to the laboratory.

Culturing Other Viscera

The ventral approach can also be used for sampling other organs, such as spleen and liver. If the peritoneal cavity has not been entered aseptically, the surface of the organ to be sampled can be seared with a hot scalpel blade and then a loop can be inserted through the seared tissue until unheated tissue is reached; this is only possible with large fish. For smaller fish, whole organs are removed aseptically, and a loop is used to streak the tissue across a plate.

Culturing Skin Lesions

Skin lesions are common in many bacterial diseases, and some bacterial diseases begin as primary skin infections. It can be difficult to determine the initiating agent because lesions are often overgrown by secondary invaders. It is important to sample early lesions whenever possible to determine the predominant organism, since the latter is often the initiating agent.

To avoid contamination of the sample, it is best to culture skin lesions on fish that have not yet had any other clinical procedures performed. Skin lesions can be cultured with a loop, but it is easier to isolate single colonies when the following procedure is used:

1. Place a sterile, 1 µl volume loop into the leading edge of the skin lesion. It can be useful to aseptically remove some scales from the edge of the lesion to be sure that the leading edge is sampled; however, this is usually not needed.
2. Immediately inoculate the material on the loop into a small, 4 mm^2 area on the periphery of a culture plate.
3. Using a sterile Mini-Tip Culturette (Becton-Dickinson), immediately swab the inoculated area onto half of the plate; then pull the streak across one quarter of the plate and then across the final quarter of the plate. This procedure almost always results in the isolation of single colonies and also allows you to estimate the number of bacteria present in the lesion (Fig. I-20).

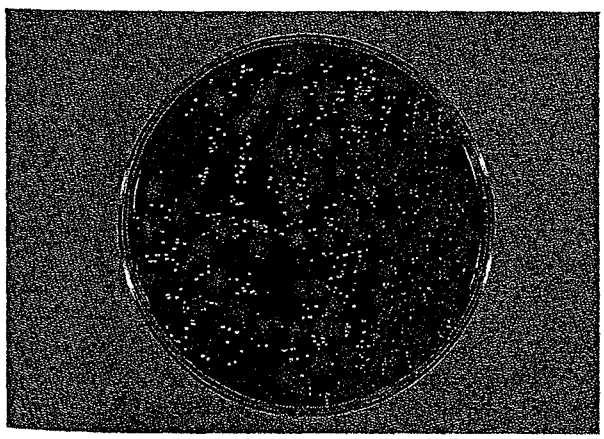

Fig. I-20 A blood agar plate from a skin lesion of a fish having well-isolated bacterial colonies.

Rapid Screening for Antibiotic Susceptibility

Bacterial infections can spread rapidly through a population, and it is important to treat fish with an appropriate antibiotic as soon as possible, since a matter of a day or two can be crucial. It can thus be useful to rapidly screen for antibiotic susceptibility. This is only a ***qualitative*** test at best and does ***not*** substitute for a properly performed sensitivity assay. However, it can provide some indication of the best antibiotic to use while the proper test is being performed. A simplified test for determining bacterial susceptibility follows (adapted from Collins, 1993):

1. Dampen the tip of a sterile swab with sterile saline. (The condensation water on the lid of a sterile bacterial culture plate can be used if it is not contaminated.)
2. Pick a single bacterial colony with the tip of the swab, and spread it as evenly as possible across the whole surface of the test medium.
3. Use a sterile forceps, and evenly distribute antibiotic sensitivity discs on the surface of the agar. Be sure the discs are firmly placed on the agar.
4. Replace the lid on the agar plate, and let it stand for a few minutes to ensure that the discs adhere. Then carefully invert the plate and incubate.
5. An inhibition zone of ≤ 15 to 16 mm suggests resistance; sensitive fish pathogens typically have clearing zones of at least 20 mm. These results may vary with the type of disc and/or antibiotic used. Testing three to four isolates is advisable.

SAMPLING FOR FUNGI

Fungal culture is rarely needed in routine fish disease diagnoses because the most common fungal pathogens, the Oomycetes (see *PROBLEM 33*), can be diagnosed without culture. However, some rarer fungal diseases (see *PROBLEM 68*) require culture for definitive diagnosis. Culture for non-Oomycetes is not usually done unless typical fungal organisms are seen either in wet mounts or via histopathology (see *PROBLEM 68*).

For fungal culture, plates should be inoculated with a small (approximately 12 mm^3) mass of fungus-infected tissue and incubated at room temperature. Once growth of the fungus is noticeable (usually within several days), it is advisable to transfer the growing edge of the mycelium to a fresh culture plate by aseptically excising a small portion of the agar containing the leading edge of growth. This procedure will help to eliminate any bacterial contaminants that were introduced with the tissue sample.

For non-Oomycetes, Sabouraud dextrose agar (SDA) is a good, commercially available, general-purpose medium for isolating almost all of these pathogens. If a non-Oomycete fungal pathogen is suspected, tissue samples should be inoculated onto SDA slants and incubated at room temperature. Be aware that airborne fungal spores can often contaminate cultures; thus, be certain that the type of fungus isolated in culture is morphologically similar to the type of fungus present in the lesions. If the fungus will not grow on SDA, other, more specialized media can be tried (see Hatai, 1989), or samples can be referred to a specialized laboratory.

Oomycetes are best isolated by using cornmeal agar, YpSs, or another nutrient-poor medium to inhibit growth of contaminating bacteria (Seymour & Fuller, 1987). While Oomycetes are usually easily isolated, culturing Oomycetes from bacteria-infected lesions may be difficult because bacteria inhibit Oomycetes, especially slow-growing forms, such as *Aphanomyces* (see *PROBLEM 34*). In heavily contaminated lesions, adding penicillin (approximately 500 U/ml) and/or streptomycin (approximately 0.2 µg/ml) may improve yields; however, some Oomycetes (especially *Aphanomyces*) are significantly inhibited by antibiotics. *Saprolegnia* and *Achlya*, the two genera most commonly isolated from fish, are usually not significantly inhibited.

SAMPLING FOR VIRUSES

Definitive identification of viral infection relies on cell culture and immunological identification of the pathogen. Such procedures are best left to competent laboratory personnel who specialize in such techniques. However, reliable use of those techniques depends upon the submission of high-quality samples. Different viruses vary in their abilities to survive preservation procedures; specific recommendations are given for specific viral diseases in the problem list. However, in general, live fish are best submitted when a virus is suspected and the specific agent is uncertain. Otherwise, fish on wet ice or dry ice should be sent immediately by overnight mail. Fish that cannot be sent immediately should usually be frozen at the lowest temperature possible, although it is best for some viruses to store samples at 4° C if processing will occur in a few days.

EXAMINING TISSUES POSTMORTEM

Circumstances permitting, it is always desirable to do a complete necropsy on selected individuals. Four to six fish showing clinical signs that are typical of the outbreak should be necropsied, if possible. While necropsy may not be possible with highly valuable fish, it is mandatory when a clinician performs an examination of a large fish population that includes expendable fish.

Condition of Tissue

The diagnostic usefulness of the postmortem examination is highly dependent upon the quality of specimens presented (see Table I-3). Whenever possible, live fish should be examined. Owners may present fish for diagnosis that have recently died; however, such fish are often of no diagnostic value. Fish decompose much more rapidly than mammals under similar conditions; this is especially true for small fish. Most ectoparasitic protozoa and Monogenea (see *PROBLEMS 16, 18*) die within minutes to hours of host death, depending on temperature and parasite species. Larger parasites, such as copepods (see *PROBLEM 13*) or branchiurans (see *PROBLEM 14*), may be detectable for longer periods.

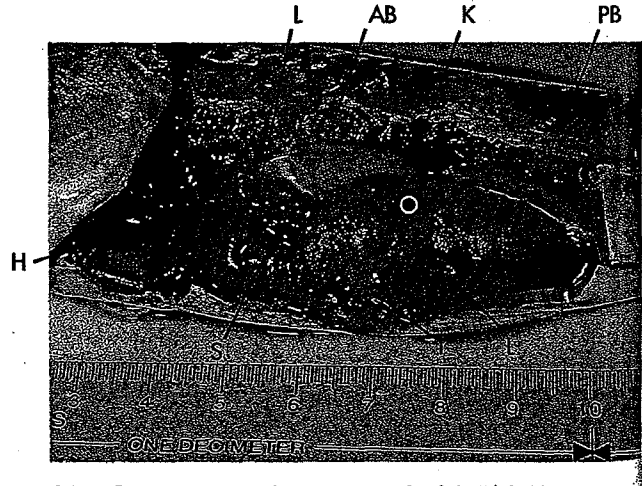

Fig. I-21 Gross anatomy of the viscera of a fish (koi). *H* = heart; *L* = liver, which has several lobes covering the intestine (*I*); *O* = ovary, which is large because this fish was almost ready to spawn; *K* = kidney; *S* = spleen. Note that the swim bladder has an anterior (*AB*) and posterior (*PB*) chamber. This is characteristic of cyprinid fish, but other fish have a single chamber.

Bacterial invasion of both skin and internal organs occurs rapidly after death, making interpretation of culture results difficult. Finally, because fish tissues autolyze rapidly, histological evaluations are compromised.

If submitting live fish is not an option, animals can be put in a plastic bag and placed on wet ice. Again, the diagnostic value of the tissues will deteriorate with time; fish should be examined within several hours of death.

If fish cannot be submitted within several hours, euthanized fish should be frozen immediately (−20° C). Most ectoparasitic protozoa and Monogenea will usually not be recognizable after freezing, but the macroscopic host response to some protozoa may be visible (e.g., white cysts of *Ichthyophthirius*).

Protozoan ectoparasites and Monogenea usually cannot be identified from wet mounts of chemically preserved (fixed) tissue. Most parasites are recognizable in histological sections, but many ectoparasites detach from the skin and gills during processing, so they may be difficult to find in sections. Granulomas (see Fig. I-33) are easily seen in wet mounts of fixed tissues. Affected tissues can then be histologically processed for a diagnosis. Histology is useful for differentiating many of the diseases affecting internal organs.

Necropsy Procedures

Skin and gill examinations should be done as described for biopsy procedures. It is often advisable not to euthanize fish until the skin and gill examinations have been completed because of the aforementioned problems with decomposition. If bacterial cultures are to be taken, these should be done next, as described previously.

After euthanization, place the fish in lateral recumbency, and make a longitudinal incision along the ventral midline from the anal opening to just ventral to the gill chamber. This incision will exend from the posterior peritoneal cavity into the pericardial sac. Make latitudinal incisions at both ends of this previous incision that extend to the dorsal aspect of the body cavity. Reflect the body wall dorsally, exposing the viscera (Fig. I-21).

If fluid is present, make smears as described for blood sampling. Identify and examine the intestines, liver, spleen, gonads, and heart. Reflect the swim bladder ventrally and examine the anterior kidney and posterior kidney (Fig. I-22). The braincase is entered by using a pair of sharp scissors to reflect the dorsal cranium anteriorly (Fig. I-23, *A* and *B*). After visual inspection, fine scissors and forceps are used to remove the brain in toto (Fig. I-23, *C*). Direct smears of various tissues can be stained for bacteria, although it is best to stain histological sections appropriately (e.g., Brown and Brenn's Gram stain) so that host response and tissue damage can also be evaluated.

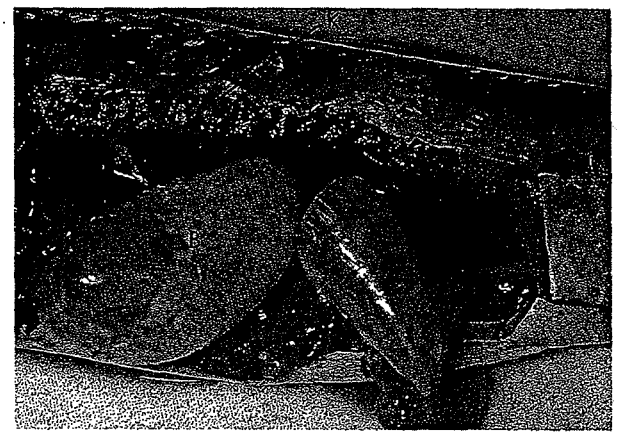

Fig. I-22 Viscera and swim bladder [*B*] in Fig. I-21 have been reflected, revealing the kidney [*K*].

Fixation Procedures

A 1 cm^2 portion of each lesion and of each organ should be placed in fixative. Even small fish should be dissected to expose internal organs to fixative, although very small fish (<5 mm or 0.2 inch) can be usually be fixed in toto without autolysis artifacts. The fixative of choice for routine diagnosis is 10% neutral buffered formalin. Bouin's fluid is considered by some to provide better fixation, but it has several disadvantages (potentially explosive when dry, difficult to remove totally from fixed tissues, and damages fixed tissues if not completely removed) that reduce its attractiveness.

Tissues can be processed routinely using standard histological techniques and embedded in paraffin (Bucke, 1989). Note that gills and scaled skin must be decalcified before sectioning. Hematoxylin and eosin and other standard stains can be used on fish tissues. Thus, samples can be submitted to mammalian histopathology laboratories.

Wet Mount Procedures

It is often useful to also make tissue squashes, especially of kidney, spleen, liver, or any lesions. Small fish can be squashed whole or the entire viscera can be removed and squashed. To make a tissue squash, excise a small (approximately 8 mm^3) piece of tissue and place it on a slide with a drop of water or normal saline. Place the edge of a plastic coverslip near the tissue, and then gently squash it (Fig. I-24). Examine the tissue architecture under low (100X) magnification and look for parasites and granulomas; then crush the tissue into a thin smear, and examine it at 100X and high dry (400X) power to identify protozoa and bacteria.

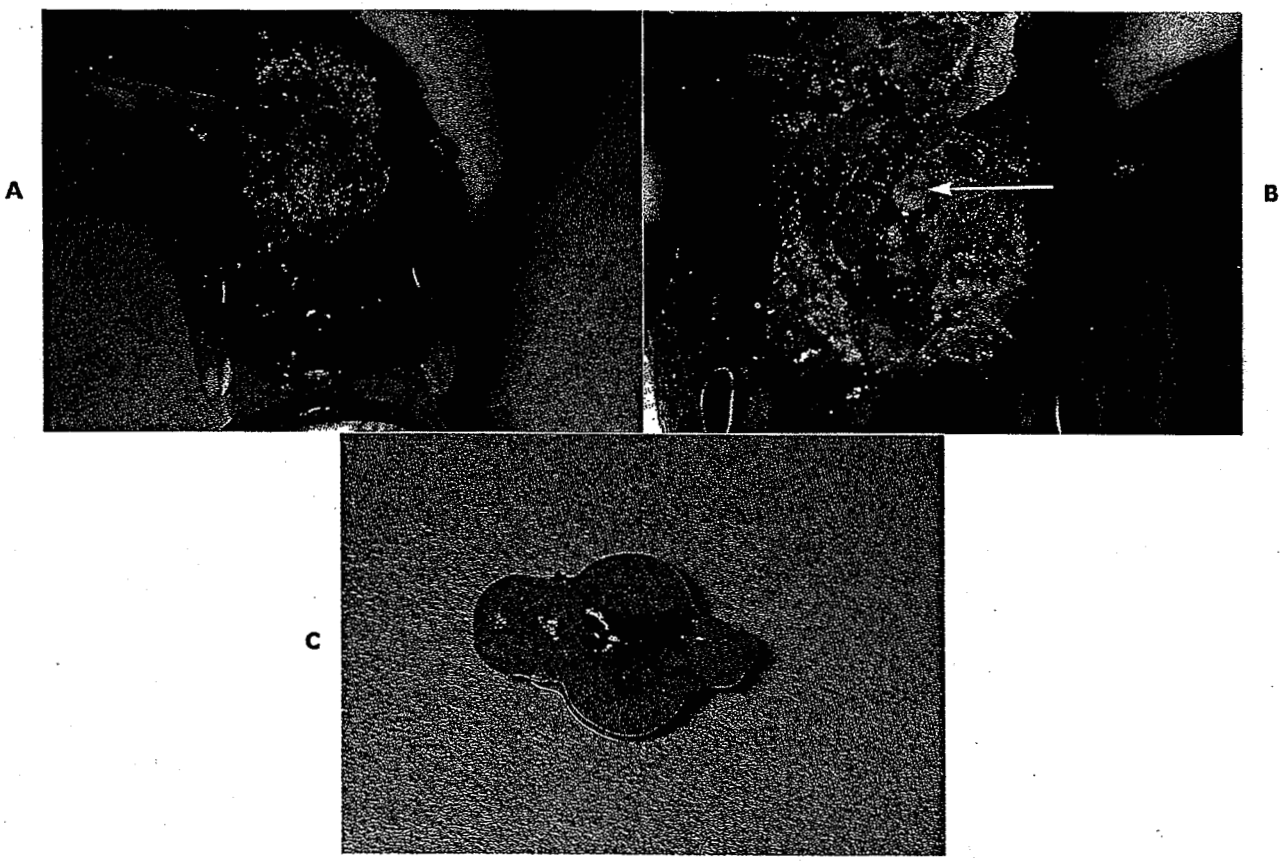

Fig. I-23 Brain necropsy. A, Exposing the brain by using rongeurs to reflect the dorsal portion of the skull posteriorly. B, The brain in situ (*arrow*). C, Intact brain removed.
(*A*, *B*, and *C* photographs by L. Khoo and E. Noga.)

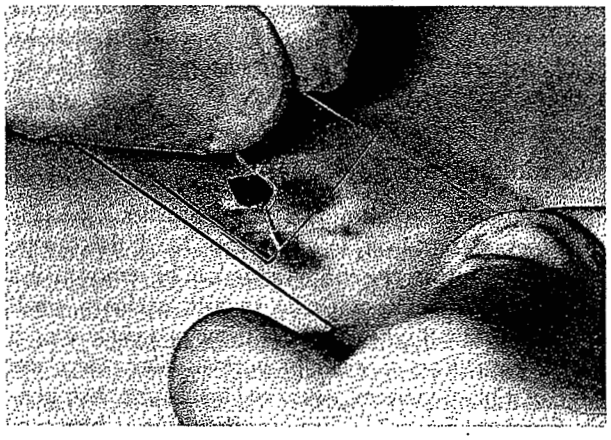

Fig. I-24 Squashing tissue for a wet mount.

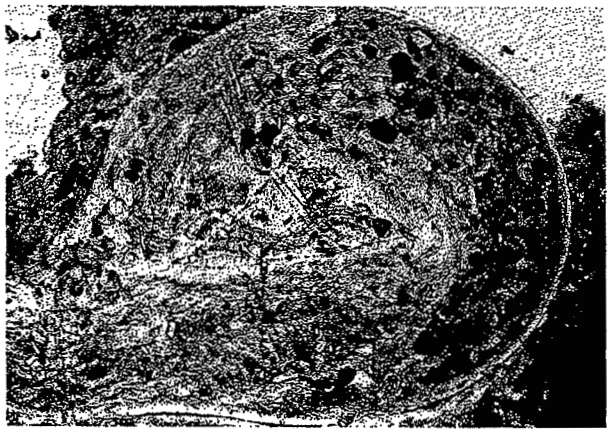

Fig. I-25 A, Wet mount of normal intestine of a small (~2.5 cm) fish. The intestine is thin-walled, and the luminal contents are easily seen.

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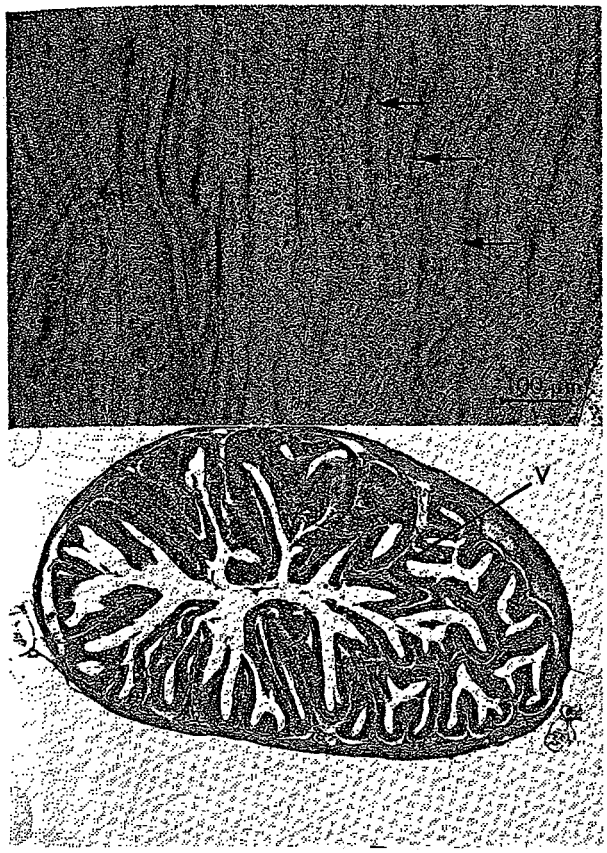

Fig. I-25—cont'd. B, Wet mount of intestine showing rugae, or folds (*arrows*), which are composed of villi. C, Histological cross-section of normal intestine. *V* = villi. Hematoxylin and eosin.
(*B* photograph by L. Khoo and E. Noga.)

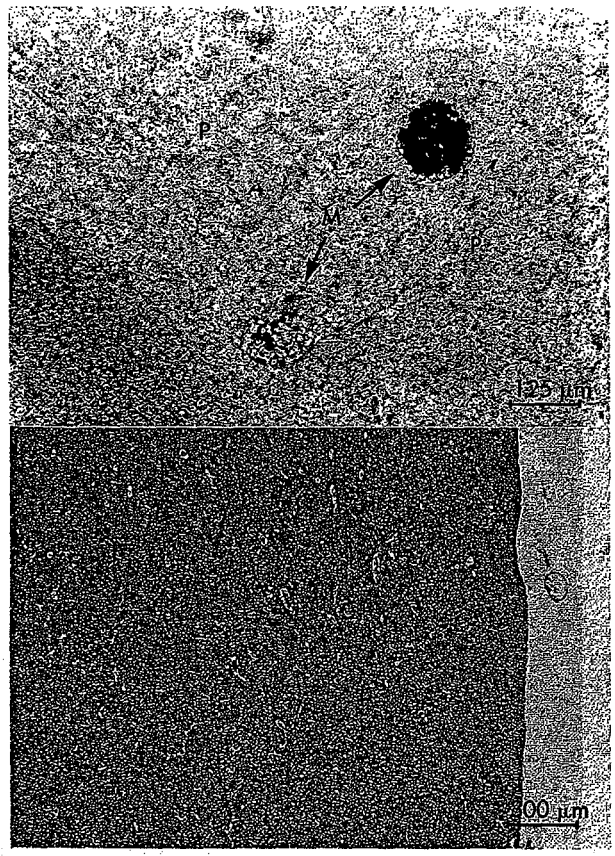

Fig. I-26 A, Wet mount of normal liver. Note homogeneous parenchyma (*P*) and aggregates of pigmented macrophages, the melanomacrophage centers (*M*). B, Histological section of normal liver. Hematoxylin and eosin.
(*B* photograph by L. Khoo and E. Noga.)

Structure of Normal Tissues

The viscera of fish are generally similar to those of mammals, but certain peculiarities should be recognized. Small fish, such as most aquarium fish, have little connective tissue stroma, making the viscera flaccid and coincidentally facilitating the preparation of wet mounts. Note that squashes are most easily made from (and thus most useful in) organs of small fish. Organs of large fish (≥ 20 to 25 cm or 8 to 10 inches) have more connective tissue and are harder to squash. Pigmented cells are a normal finding in virtually all organs and are especially common in hematopoietic tissues. The peritoneum of many fish is lined with melanocytes. Aggregates of pigmented cells, the melanomacrophage centers, are also common (see Fig. I-27).

Key Features of Internal Organs

Intestine — The intestinal tract is usually the first organ seen when the peritoneal cavity is opened. However, body fat is most commonly deposited in the peritoneal cavity and may obscure the viscera. The intestinal tract is a straight, thin-walled tube. In many aquarium fish the lumen is too small to be easily cut open, but in such fish the intestinal contents can often be seen through the wall (Fig. I-25, *A* through *C*). The intestine should be opened ***after*** the other viscera have been examined to reduce contamination by bacteria and other organisms. The stomach is larger than the intestines. The presence or absence of food in the intestinal tract is easily assessed.

Liver — The liver is a brown to red-brown to tan organ in the anterior portion of the peritoneal cavity. Microscopically, normal liver has a homogeneous appearance; an occasional melanomacrophage center may be seen (Fig. I-26, *A* and *B*).

Gall Bladder — The gall bladder is a large, translucent sac with green or yellowish fluid. It lies close to the liver and is often large (i.e., it is often larger than the spleen).

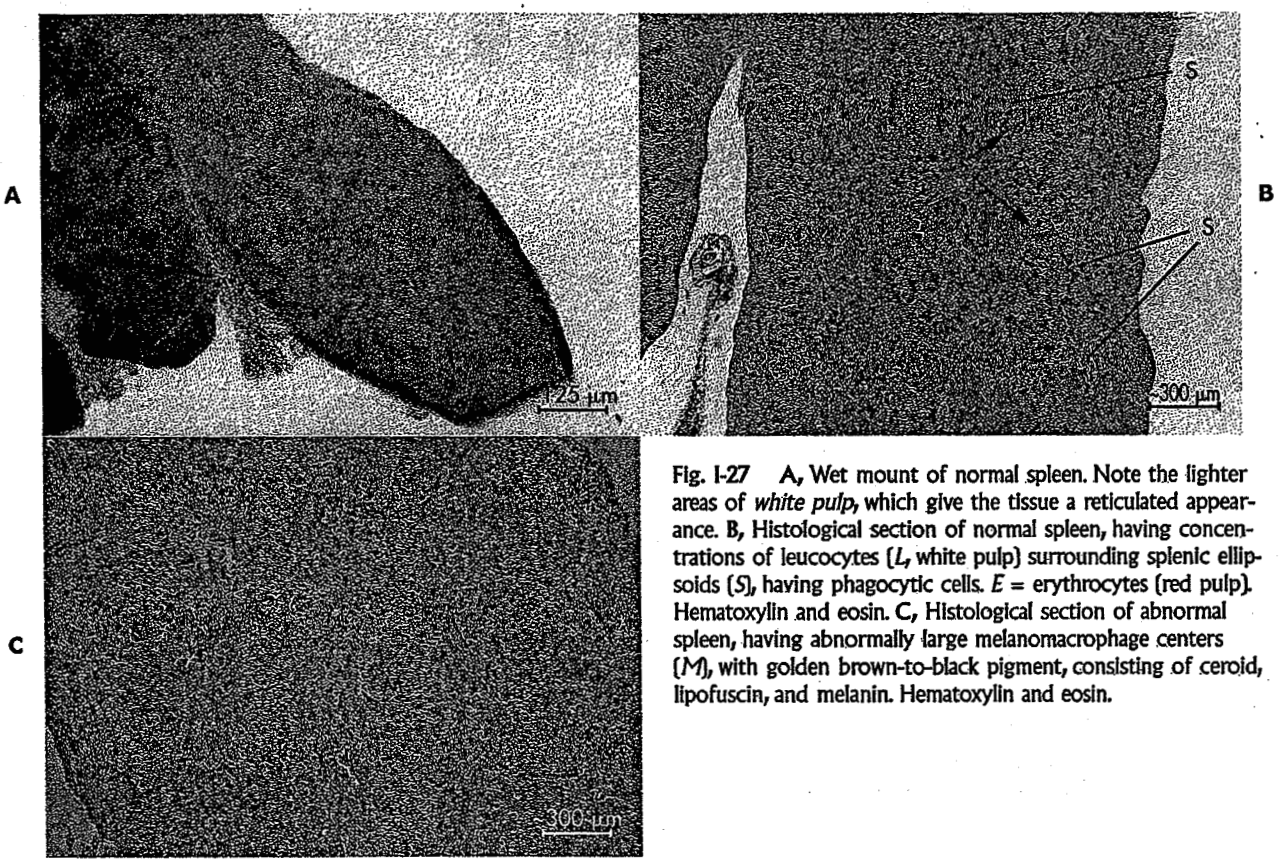

Fig. I-27 A, Wet mount of normal spleen. Note the lighter areas of *white pulp*, which give the tissue a reticulated appearance. B, Histological section of normal spleen, having concentrations of leucocytes (*L*, white pulp) surrounding splenic ellipsoids (*S*), having phagocytic cells. *E* = erythrocytes (red pulp). Hematoxylin and eosin. C, Histological section of abnormal spleen, having abnormally large melanomacrophage centers (*M*), with golden brown-to-black pigment, consisting of ceroid, lipofuscin, and melanin. Hematoxylin and eosin.

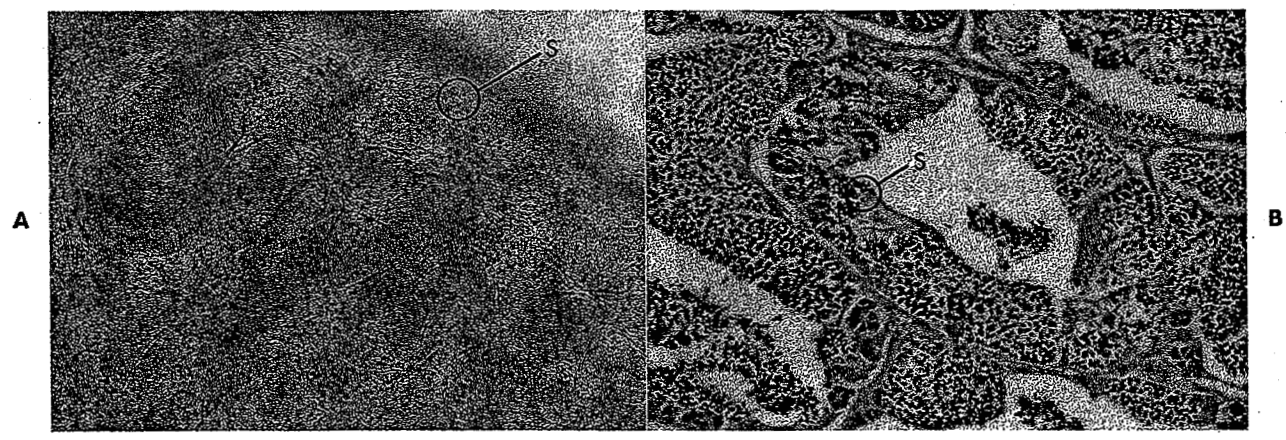

Fig. I-28 A, Wet mount of normal testis. Note individual spermatozoa (*S*) visible on the edge of the cut tissue. B, Histological section of normal testis filled with spermatozoa. *S* = spermatozoa. Hematoxylin and eosin.

(*A* and *B* photographs by L. Khoo and E. Noga.)

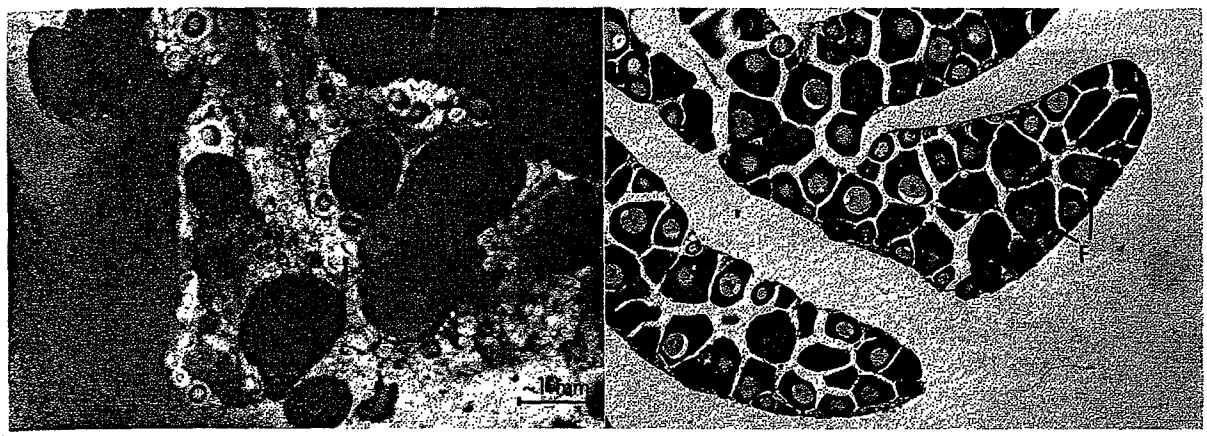

Fig. I-29 A, Wet mount of normal ovary. Compare with testes (Fig. I-28, A and B). Do not confuse follicles (F) with granulomas (see Fig. I-33). B, Histological section of normal ovary. F = follicles. Hematoxylin and eosin.

(*A* photograph by L. Khoo and E. Noga.)

It may be accidentally ruptured when the peritoneal cavity is opened, tainting the viscera yellow-green.

Spleen — The spleen is a bright red to black organ located in the mesentery. Microscopically, normal spleen has a reticulated appearance because of the network of ellipsoids that are the sites of blood filtration (Fig. I-27, *A* through *C*).

Gonad — The reproductive organs may be difficult to see in fish that are not sexually mature. In immature fish, the reproductive organs are ribbon-like, grey-white or yellow strips that usually lie just ventral to the swim bladder. In some fish that are ready to spawn, the ovaries may occupy most of the peritoneal cavity and cause gross abdominal distension. Even in immature fish, sex can often be determined by examining a wet mount, which may reveal the presence of sperm (Fig. I-28, *A* and *B*) in a male or follicles (Fig. I-29, *A* and *B*) in a female.

Swim Bladder — The swim bladder is a white, shiny organ that lies near the back (dorsum), just ventral to the kidney. Filled with gas, its primary function is to maintain buoyancy.

Kidney — The kidney is a retroperitoneal organ that is functionally (and often morphologically) divided into two segments. The anterior kidney is the primary site of hematopoiesis; it is a dark red to black, soft amorphous tissue that has the consistency of bone marrow (Fig. I-30, *A* and *D*). The posterior kidney has a similar gross appearance but has renal excretory tissue as well (Fig. I-30, *B, C,* and *E*).

Heart/Skeletal Muscle — The heart lies in the pericardial cavity, which is just anterior to the peritoneal cavity in the *throat* region of the fish. It is a red, highly muscular, two-chambered organ. It empties into the ventral aorta via the white, elastic, bulbus arteriosus. Wet mounts of normal skeletal or cardiac muscle will reveal individual muscle fibers with striations (Fig. I-31).

Brain — The brain is superficially similar to those of mammals, with morphological differentiation of various neural centers. Microscopically, it appears as a grey-white organ that has an amorphous appearance on wet mount.

Glands — Most of the major glandular tissues found in mammals occur in fish; they are only detectable histologically, except for thymus (Fig. I-32). Analogues of the adrenal cortex (interrenal cells) and adrenal medulla (chromaffin cells) are found in the anterior kidney. Pancreatic exocrine and endocrine tissues are usually dispersed throughout the mesentery or may be associated with the liver or spleen. Thyroid tissue is usually dispersed around the ventral aorta but may also be found in the kidney, spleen, or mesentery.

Common Lesions Found in the Viscera

Necropsy can provide information on nutritional status. Aquarium fish are often overfed, resulting in excessive accumulation of fat in the peritoneal cavity. In fish that are fed unbalanced diets, the liver may be pale yellow because of lipidosis. The significance of obesity in pet fish is uncertain, but excessive lipid deposition is commonly associated with clinical disorders in food fish, such as trout (see *PROBLEM 79*). However, note that normal liver color varies considerably among species; it also varies seasonally, so it is necessary to be aware of the normal physiological color variation for a particular species.

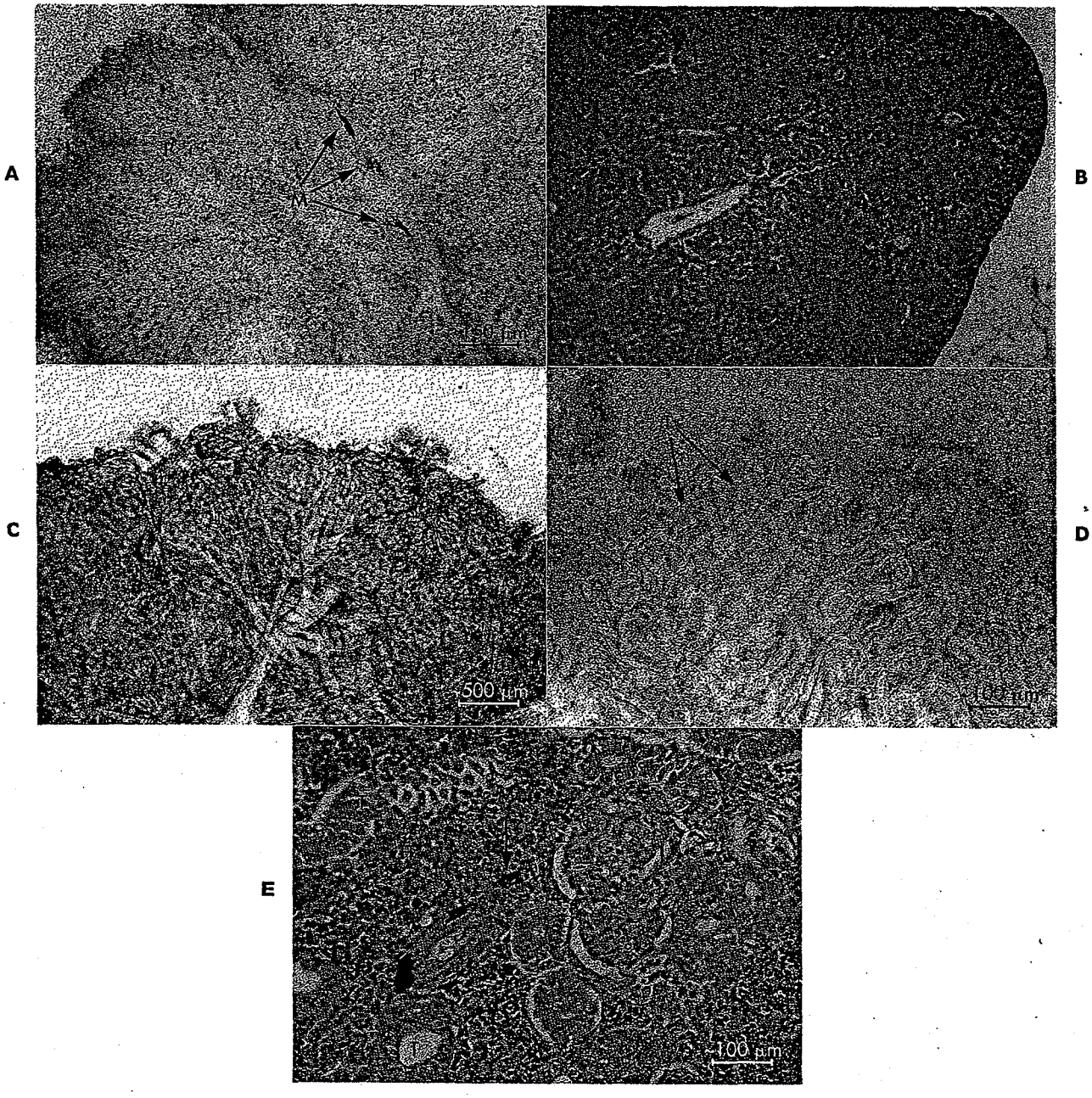

Fig. I-30 A, Wet mount of normal anterior kidney. Note the homogeneous parenchyma (*P*). Most of the cells are hematopoietic elements. The absence of renal excretory tissue is a normal finding. *M* = melanocytes. B, Histological section of normal anterior kidney. Hematoxylin and eosin. C, Wet mount of normal posterior kidney. Low-power view showing dendritic collecting duct. D, Wet mount of normal posterior kidney. Higher-power view, showing renal tubules (*T*). Most of the interstitial tissue surrounding the tubules is hematopoietic. E, Histological section of normal posterior kidney. *G* = glomeruli; *T* = renal tubules; *H* = hematopoietic tissue; *M* = melanocyte. Hematoxylin and eosin.

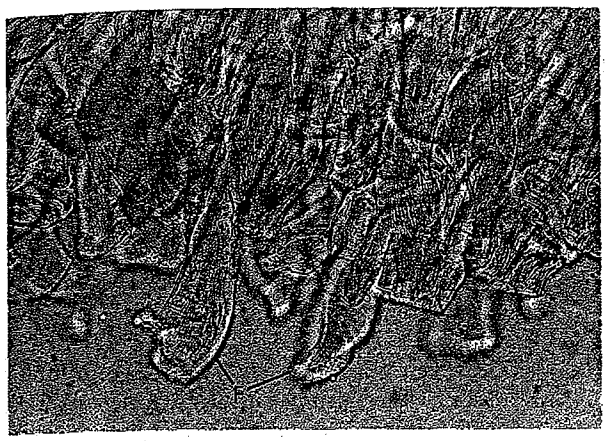

Fig. I-31 Wet mount of normal skeletal (striated) muscle. Note the individual fibers (*F*) with striations.

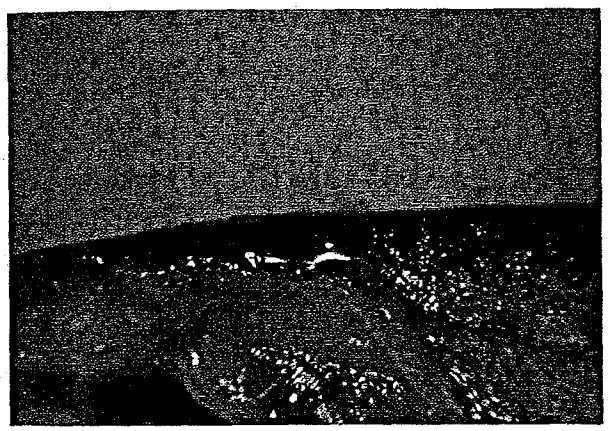

Fig. I-32 Thymus (*T*), located at the dorsomedial aspect of the gill chamber. Head is to the left. *P*=pseudobranch.

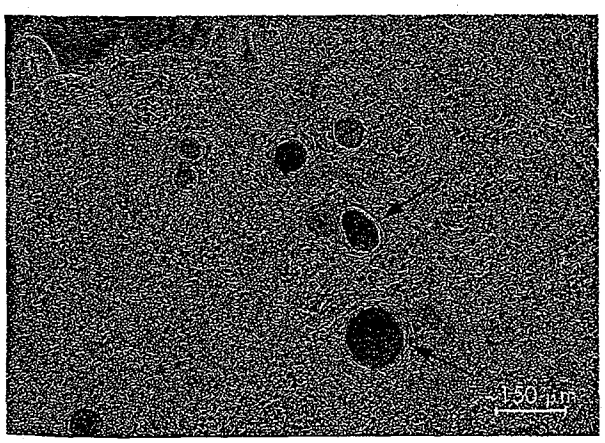

Fig. I-33 Granulomas in a wet mount. Note the dark, necrotic center (*C*) surrounded by lighter, viable, inflammatory cells (*arrows*).

Fluid accumulation in the abdomen (*dropsy*) (see Fig. I-3, *B*) is a common clinical presentation. It can result from infection by viruses, bacteria, or parasites. Examination of abdominal fluid may reveal bacteria or parasites (e.g., *Hexamita*). Ascitic fluid may also form from osmoregulatory dysfunction. Hemorrhages in the viscera can be caused by systemic viral or bacterial infections.

Several chronic inflammatory diseases can affect internal organs. Among the most important is mycobacteriosis, which can affect virtually any internal organ. Granulomas produced by this pathogen must be differentiated from neoplasia (see *PROBLEM 72*) (foreign-body reactions produced against protozoan or metazoan parasites) and from melanomacrophage centers.

Melanomacrophage centers (MMC) are usually solid foci of cells that have varying amounts of pigment (see Fig. I-27). While these are common in healthy fish, they increase in number with chronic stress (Wolke, 1992). Thus, MMC are an indicator of chronic stress; however, it is necessary to know the normal prevalence in a particular fish species to make an accurate diagnosis of chronic stress.

In contrast to melanomacrophages, granulomas are usually multilayered structures having a central zone of necrotic debris (Fig. I-33). This necrotic center is the most useful feature for identifying granulomas. It is important to recognize that granulomas may contain pigment and melanomacrophage centers accumulate in many disease states. Thus, in some cases, histology may be needed for differentiation, especially if other tests are negative.

Trematodes, nematodes, and cestodes, especially larvae, occur in the mesentery or viscera. Compared with mammals, internal helminths are much less serious problems in fish. However, some internal helminths can cause serious disease.

ZOONOTIC DISEASES

Several zoonotic helminths can infect humans but can only be contracted after ingestion of infected fish. A few fish pathogens can infect the clinician during a clinical work-up. Aeromonads, vibrios, and *Edwardsiella tarda* (see *PROBLEMS 45, 48,* and *49*) can infect the skin or cause gastroenteritis or systemic infections. However, the agent of most concern is *Mycobacterium* (see *PROBLEM 53*). Only atypical mycobacteria infect fish; these are the least pathogenic mycobacteria for humans, but they can cause *fish tank granuloma*, a chronic infection that is usually limited to the extremities (i.e., fingers and hands). Fortunately, incidences of zoonotic infections with these pathogens appear to be uncommon events when compared with the relative risk of exposure to these agents. However, appropriate caution is warranted, especially in immunosuppressed individuals (Angulo et al., 1994).

CHAPTER 6

Guidelines for Interpreting Clinical Findings

STRESS AND FISH DISEASE

The metabolic, biochemical, and physiological processes of fish are basically similar to those of mammals. Fish are susceptible to the same types of agents that affect warm-blooded animals, including viruses, bacteria, fungi, parasites, as well as various noninfectious problems. However, stress appears to play a considerably larger role in causing disease in fish (Walters & Plumb, 1980; Collins et al., 1976). Stress can be considered as a continuum of insults, varying from mild to severe (Fig. I-34). How much of an impact stress has on a fish depends on the severity of the stress, its duration, and the physiological state of the fish, among other considerations. Thus, many disease problems in fish stem from poor management; this important principle should always be kept in mind when trying to identify the true cause of a fish disease.

ENVIRONMENT AND FISH HEALTH

Good water quality is the key to successful fish production. Water quality includes all physical, chemical, and biological factors that influence the use of water for fish culture. Any characteristic of water that affects the survival, reproduction, growth, or management of fish is a water-quality variable. An abundant water supply solves many problems associated with intensive fish culture by diluting out accumulated wastes and toxic products, as well as by maintaining optimal water conditions. However, water is a precious and often limiting resource in aquaculture, and thus many methods have been developed to increase the holding capacity of culture systems.

ACCLIMATION: ITS RELATIONSHIP TO INTERPRETING WATER QUALITY AND OTHER ENVIRONMENTAL INFLUENCES

Acclimation is the physiological adaptation of an animal to a new environment. Acclimation is an important concept to understand in fish health because it helps explain why fish may get sick under one set of circumstances but may be perfectly healthy under exactly the same conditions at some other time.

A tank of fish in which the pH has slowly dropped from 7.0 to 5.5 over several months may appear normal; however, if the water is rapidly adjusted back to 7.0, many of the fish may die. Even though pH 5.5 is stressful and not healthy, many fish can tolerate such conditions if they are introduced to the environment slowly. Whereas, even though a pH of 7.0 is within the normal range for most freshwater fish, too rapid a return to normal will be dangerous.

With the chronic low pH stress described above, where environmental conditions gradually deteriorate, indirect effects of the stress are often seen; these may include failure to reproduce, poor growth, developmental anomalies,

Relationship between environmental stress and the health status of fish

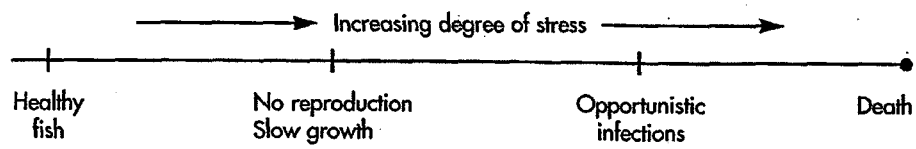

Fig. I-34 Relationship between environmental stress and fish health.

or, commonly, the presence of what are referred to as opportunistic infections; that is, diseases that develop when the fish's defenses are not up to par. As you review the problems in the diagnostic guide, you will notice that most of the environmental (water quality) problems often occur concurrently with opportunistic infections. Most infectious diseases of fish probably take advantage, in one way or another, of compromised defenses; however, some pathogens readily do this. These particular agents are generally considered to have a relatively low pathogenicity for fish and thus can only flourish under immunocompromising conditions. Classical examples of such pathogens include the bacteria *Aeromonas hydrophila* (see *PROBLEM 45*) and flexibacteria (see *PROBLEM 36*), water molds (see *PROBLEM 33*), and the parasites *Trichodina* (see *PROBLEM 21*) and ectocommensal protozoa (see *PROBLEMS 31* and *32*). When such pathogens or other opportunists are encountered, look closely for a primary environmental cause.

Inability to acclimate explains why fish often become sick after being handled or transported. The stress created by handling, combined with exposure to new environmental conditions (see *PROBLEM 86*), can cause severe stress against which fish cannot compensate.